Targeting Tumor Microenvironment by Zoledronate as a Novel Therapeutic Approach in Cancer

Dissertation
zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Sibel Mete

aus
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Promotionskomitee

Prof. Dr. Reto Schwendener (Leitung der Dissertation)
Prof. Dr. Anne Müller (Vorsitz)
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-dedicated to my lovely parents-

-sevgili anne ve babama adanmıştır-

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1. SUMMARY

Zoledronate is a drug that inhibits osteoclastic bone resorption and is commonly used to prevent and treat osteoporosis. Recently, due to preclinical evidence of its antitumoral properties, there is emerging interest in use of zoledronate as an anticancer agent. In view of its high affinity for bone matrix, studies employing *in vivo* tumor models largely addressed the ability of zoledronate to reduce skeletal tumor burden and prevent bone metastases. However, whether clinically relevant doses of the drug prevent tumor progression in soft tissue tumors and what the corresponding mechanisms of its antitumor effects might be, are still areas of investigation.

To address these issues, immunocompetent mice bearing syngeneic subcutaneous tumors were treated with zoledronate at doses equivalent to those recommended for humans. A significant reduction in growth of LLC and MC38 tumors was observed in zoledronate-treated mice. Tumors in treated animals showed an increase in apoptotic cell death, accounting for the decreased tumor growth rate. Although several *in vitro* studies have reported a direct pro-apoptotic effect of zoledronate on cancer cells, *in vivo* achievable concentrations of the drug are not cytotoxic to cancer cells. Therefore, we examined the effect of zoledronate on the tumor microenvironment, with a focus on tumor-infiltrating myeloid cells that are in a privileged position to internalize the drug. Surprisingly, flow cytometry analysis revealed an increase in the frequency of $CD11b^+$ myeloid cells in the tumors as well as in the spleen, blood and peritoneum of treated animals. It has long been recognized that solid tumors actively recruit myeloid cells and skew their differentiation toward an immunosuppressive and pro-tumorigenic phenotype, the so-called M2-like phenotype that has been implicated in regulating many of the "hallmarks of cancer" and thus fostering solid tumor development. Therefore, the unexpected inverse correlation between myeloid cell density and tumor growth in zoledronate-treated animals prompted us to examine the activation status of these cells in treated animals. To this end, tumor-infiltrating myeloid cells were isolated and their activation status was assessed by q-PCR analysis of relevant markers of M1 and M2 activation states. Differential expression analyses revealed that myeloid cells from treated

tumors regained an M1-type antitumoral phenotype, showing an increased expression of proinflammatory and immunostimulatory mediators such as IL-12, iNOS and IFN-γ and a reduced expression of the immunosuppressive factors IL-10, arginase and VEGF. Furthermore, these cells displayed an enhanced ability to stimulate $CD8^+$ T cell proliferation and IFN-γ production.

Further characterization of the $CD11b^+$ myeloid cells identified neutrophils as the increasingly accumulating myeloid cell type in tumors of zoledronate treated animals. Accordingly, treated tumors displayed increased production of the neutrophil-attracting chemokines CXCL2/MIP-2 and CXCL5/LIX. To further identify the cellular targets of zoledronate action in tumors, two major myeloid subsets, namely $CD11b^+Ly6G^+$ neutrophils and $CD11b^+Ly6G^-$ monocytes/macrophages, were sorted and analyzed for changes in their immunostimulatory and immunosuppressive properties. Zoledronate induced a proinflammatory and antitumorigenic activation profile specifically in the neutrophil, but not the macrophage subset of myeloid cells. Study of pharmacological inhibition of neutrophils confirmed that neutrophils are essential for the antitumorigenic effects of zoledronate, as their depletion renders zoledronate ineffective in restricting tumor growth. Another interesting finding was that administration of recombinant TGF-β to zoledronate-treated animals reduces the therapeutic efficacy of the drug by impairing the increased neutrophil influx in tumor. Accordingly, in simplified cell culture-based assays, we showed that TGF-β levels influence the neutrophil chemotaxis towards tumor cells derived factors.

Finally, we attempted to improve the antitumor efficacy of zoledronate by encapsulating the drug in liposomes. Liposomal encapsulation significantly improved the efficacy of zoledronate by altering its pharmacokinetics and biodistribution profile.

Collectively, our findings reveal novel antitumorigenic properties of zoledronate that may serve as a basis for the design of more effective immunotherapeutic approaches against cancer.

1. ZUSAMMENFASSUNG

Zoledronat ist ein Wirkstoff der die Knochenresorption durch Osteoklasten inhibiert und zur Prävention und Therapie von Osteoporose eingesetzt wird. Neuere präklinische Daten zeigen, dass Zoledronat auch als Krebsmittel wirksam ist. Wegen seiner hohen Affinität zu Knochengewebe konzentrieren sich die meisten *in vivo* Untersuchungen mit Zoledronat vor allem auf die Reduktion von Knochentumoren und Metastasen. Ob jedoch klinisch relevante Dosen von Zoledronat die Bildung von Weichteiltumoren verhindern und durch welche Wirkmechanismen Zoledronat das Tumorwachstum blockiert ist Gegenstand weiterer Untersuchungen.

Um diese Fragen zu klären haben wir immunkompetente, tumortragende Mäuse mit klinisch relevanten Dosen von Zoledronat behandelt. Dabei resultierte eine signifikante Reduktion des Wachstums von subkutan implantierten Lewis Lungenkarzinom und MC38 Kolonkarzinom Tumoren. Behandelte Tumore wiesen erhöhte Apoptose auf, was zu reduziertem Tumorwachstum führte. Obwohl verschiedene *in vitro* Studien gezeigt haben, dass Zoledronat einen direkten pro-apoptotischen Effekt auf Tumorzellen ausübt, sind die *in vivo* erreichbaren Konzentrationen nicht zytotoxisch auf Tumorzellen. Deshalb untersuchten wir den Effekt von Zoledronat auf die Tumormikroumgebung und hauptsächlich auf die Tumor infiltrierenden myeloischen Zelltypen, die Zoledronat bevorzugt aufnehmen. Ueberraschenderweise ergab eine durchflusszytometrische Analyse, dass die Frequenz von CD11b positiven myeloischen Zellen nach Behandlung mit Zoledronat in Tumoren aber auch in der Milz, im Blut und im Peritoneum stark erhöht war. Es ist bekannt, dass solide Tumore myeloische Zellen aktiv rekrutieren und deren Differenzierung in immunosuppressive und das Tumorwachstum fördernde, sogenannte M2-Zelltypen verursachen. Diese Zellen regulieren verschiedene Prozesse im Tumorwachstum und sind daher für die Entwicklung von soliden Tumoren ausserordentlich wichtig.

Die unerwartete inverse Korrelation zwischen der Zelldichte der myeloischen Zellen und dem Tumorwachstum bei Zoledronat behandelten Tieren bewog uns den Aktivierungsstatus dieser Zellen näher zu untersuchen. Dazu wurden tumorinfiltrierende

myeloische Zellen isoliert und der Aktivierungsstatus von relevanten M1- und M2-spezifischen Genprodukten mittels quantitativer Polymerasenkettenreaktionsanalyse untersucht. Diese Analyse ergab, dass myeloische Zellen von behandelten Tumoren vom M1-Phenotyp waren, mit erhöhter Expression von pro-inflammatorischen und immunostimulatorischen Faktoren wie IL-12, iNOS und γ-Interferon, sowie verminderter Expression der immunosuppressiven Faktoren IL-10, Arginase und VEGF. Diese Zellen hatten auch die Eigenschaft, CD8 positive T-Zellen zu stimulieren und die γ-Interferon Produktion zu erhöhen. Die weitere Charakterisierung der CD11b positiven myeloischen Zellen identifizierte Neutrophile als der Zelltyp, der in Zoledronat behandelten Tumoren angereichert wurde.

Dementsprechend konnte in behandelten Tumoren eine erhöhte Produktion der Neutrophil rekrutierenden Chemokine CXCL2/MIP-2 und CXCL5/LIX nachgewiesen werden. Um die zellulären Zielmoleküle von Zoledronat weiter zu identifizieren wurden die beiden wichtigsten myeloischen Untergruppen, nämlich die $CD11b^{+}Ly6G^{+}$ Neutrophilen und die $CD11b^{+}Ly6G^{-}$ Monozyten/Makrophagen sortiert und auf ihre immunostimulatorischen oder -suppressiven Eigenschaften untersucht. Zoledronat induzierte ein pro-inflammatorisches und tumorsuppressives Aktivierungsprofil in den Neutrophilen, jedoch nicht in den Monozyten/Makrophagen. Die pharmakologische Inhibition der Neutrophilen bestätigte, dass dieser Zelltyp für die antitumor Effekte von Zoledronat verantwortlich waren, da ihre Depletion die Wirkung von Zoledronat aufhob. Ein weiteres interessantes Resultat war, dass rekombinantes TGF-β den therapeutischen Effekt von Zoledronat durch Verminderung der Akkumulation von Neutrophilen ins Tumorgewebe aufhob. Entsprechende vereinfachte Experimente in Zellkulturen bestätigten, dass TGF-β die Chemotaxis von Neutrophilen zu tumorzellspezifischen Faktoren beeinflusst.

Schliesslich wurde die antitumor Wirkung von Zoledronat durch Einkapselung in Liposomen optimiert. Liposomales Zoledronat war in signifikanter Weise besser wirksam, indem dessen pharmakokinetisches Profil verändert wurde.

Zusammenfassend zeigen unsere Resultate neue Eigenschaften von Zoledronat, die die Entwicklung von effektiveren immunotherapeutischen Tumortherapien ermöglichen.

2. INTRODUCTION

2.1 Tumor development and heterogeneity in the tumor microenvironment

Although cancer is a systemic disease, it originates from a single cell harboring a mutant DNA sequence that reroutes the crucial pathways regulating survival and death of the cell.[1] Survival advantaged mutant cells neutralize further homeostatic growth constraints and keep proliferating, and continue accumulating other genetic alterations, including chromosomal abnormalities and mutations in the sequence of DNA. Due to genomic instability and high proliferation rates, each cancer cell has a unique mutational signature that gives rise to genetic heterogeneity of a tumor (Figure 2.1).[2] For the majority of tumors, genetic heterogeneity confers a growth advantage upon cancer cells and this heterogeneity is also implicated in the development of drug resistance in cancer therapy. This hypothesis has been supported by clinical observations showing that less heterogeneous tumors like chronic myeloid leukemia (CML) that are driven by a common genetic aberration show better responses to treatment compared to highly heterogeneous tumors types like breast and ovarian cancers. [3-5]

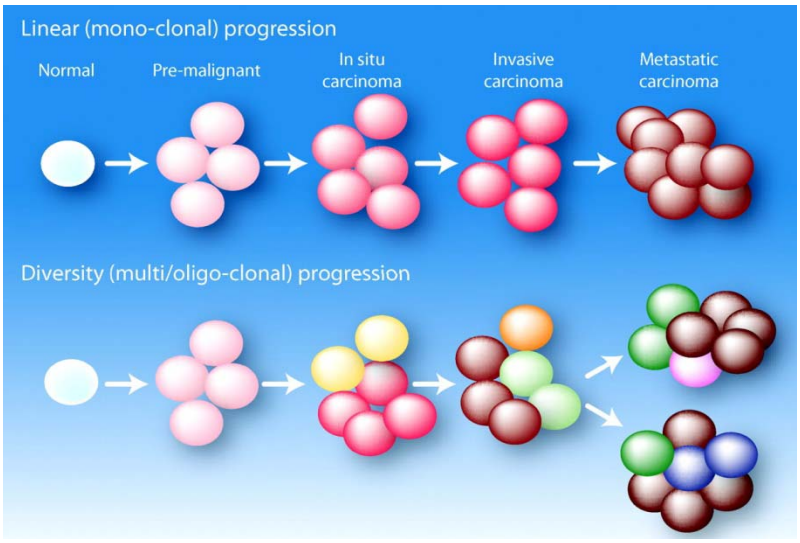


Figure 2.1: Models of mono-clonal and multi-clonal (heterogeneous) tumor progression. In some cancers there is a dominant tumor cell clone that progressively accumulates and inherits similar genetic and epigenetic alterations. On the other hand, there could be a predominant clone within other types of tumors and there are multiple other clones present as well. Different color circles indicate distinct clones. Adapted from Polyak, 2008.[6]

In growing tumors, another type of heterogeneity which provides a growth and survival advantage to the cell is developed at the cellular level. As the cancer cells proliferate and take up more space, they begin to be restricted by environmental factors such as lack of oxygen, nutrients and pressure of the nearby tissue. Without a steady supply of nutrition and oxygen, tumors can grow only to a few thousand cells in number or measure around 1–2 mm^3.[7] These microscopic colonies of cancer cells are known as *in situ* tumors ("carcinoma in situ", CIS) and do not produce any symptoms until they are invading the surrounding tissue. Indeed, most people have small, *in situ* tumors that can remain dormant indefinitely.[8] To progress further and develop into invasive carcinoma, tumors require microenvironmental support. Solid tumors provide this support in their environment by forming distinct tissue-like structures mimicking normal tissue structure and function. Accumulated clinical evidence, in combination with various mechanistic studies, also support the contention that cancer progression mostly depends on the ability of cancer cells to hijack and exploit the normal physiological processes of the host. To this end, tumor cells send out chemical signals to modulate the different populations of the host's healthy cells.[9,10] Tumor-derived chemical signals activate genes in the surrounding healthy tissue while recruiting blood-derived immune cells simultaneously. As the tumor develops, these surrounding and recruited normal cells co-evolve into an activated state through a continuous paracrine communication, thereby creating a microenvironment that promotes cancer growth and ultimately leads to metastatic disease.[11] In this microenvironment, cancer cells mainly recruit host blood vessels, co-opt neighboring fibroblasts, and shape the local immune response to establish the essential conditions for their survival and success.[12] Thereby, malignant cells create a tolerant neighborhood in which they can function with limited interference. Collectively, connective tissue, extracellular matrix, and the resident non-cancerous cells surrounding cancer cells are known as tumor stroma. Many researchers prefer the broader term "tumor microenvironment," instead of "stroma," as it includes infiltrating cells of the immune system (innate and adaptive), and cell-free molecules (proteases and growth factors) in addition to the residential stromal components (Figure 2.2).[13] Although the same basic building blocks constitute the stroma of all tumors, the actual composition of the tumor microenvironment is quite variable, with differences seen

between individuals, between different tumor types, and in different areas of the same tumor. Furthermore, the composition of the tumor microenvironment is also altered as the disease progresses.[14] However, major differences in the stroma of different tumors are primarily quantitative. In some desmoplastic tumors, such as many carcinomas of the breast, stomach and pancreas, the stroma constitutes up to 90% of the total tumor mass. On the other hand, there are tumors such as medullary and lobular carcinomas of the breast and many lymphomas in which only minimal stroma is deposited.[15]

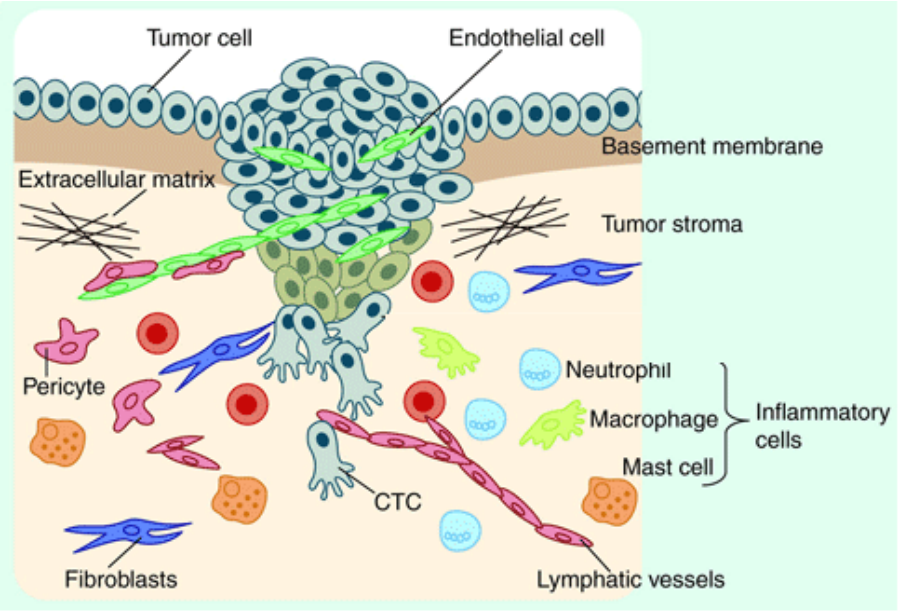


Figure 2.2: Cellular heterogeneity of a tumor. Cancer cells in solid tumors are surrounded by a complex microenvironment comprising various cells including endothelial cells of the blood and lymphatic circulation, stromal fibroblasts and bone marrow-derived cells including macrophages, myeloid-derived suppressor cells (MDSCs), neutrophils and mesenchymal stem cells (MSCs). Adapted from Jabado et al., 2007.[16]

2.1.1 Significance of the tumor microenvironment in cancer therapy

Early evidences about the effects of the environment on tumor growth were provided in 1976 by the work of Solt and Farber demonstrating that growth of a tumor from a single mutated cell can only occur when the stromal environment is altered in such a way

to allow unrestrained tumor growth.[17] In spite of this and many other significant findings, cancer research has largely focused for many years on cancer-cell driven carcinogenesis with particular emphasis on understanding the mutations causing neoplastic cell transformations. Such a cancer cell-centric view of tumor progression largely ignored the fact that complex interactions between the cancer cells and stromal cells tightly regulate growth of tumors. Partially for this reason, even after decades of implementing treatments that selectively target the tumor cell, the survival time for metastatic cancer patients is still quite short. Novel strategies are therefore urgently needed to complement and improve classical treatments for cancer. In this regard, interactions between cancer cells and their host environments offer novel opportunities for therapies based on the improved understanding of the nature of these interactions and the mechanisms that govern them. Drugs targeting both cancer cells and stromal components are likely to be more effective than those directed solely against cancer cells. A potential advantage in targeting the non-malignant cells of the tumor microenvironment is that these cells tend to be more genetically stable than cancer cells, and are therefore less likely to develop resistance to therapies.

Current strategies for cellular, gene, and molecular therapies are focusing on the manipulation of the different components of the tumor microenvironment. In this context, newly formed blood vessels have been one of the most common targeted components of the tumor microenvironment. Various monoclonal antibodies (like bevacizumab; Avastin®) and tyrosine kinase inhibitors (like SU5416, PTK787 and Sorafenib) were developed against the pro-angiogenic factor vascular endothelial growth factor (VEGF) and VEGF receptor signaling, respectively.[18] Although angiogenesis inhibitors targeting the VEGF signaling pathway have proven to be efficacious in reducing tumor angiogenesis and thereby providing a survival benefit in many preclinical cancer models as well as in clinical trials, emerging studies have demonstrated certain drawbacks of these anti-angiogenic therapies. The VEGF inhibitors having an initial anti-tumor effect in mouse models of pancreatic carcinoma and glioblastoma were found to concomitantly elicit the tumor's adaptation to anti-angiogenic therapy and increase tumor invasion and metastasis as well.[19]

As the most abundant cell types in the stroma of several solid tumors, cancer associated fibroblasts (CAFs) were recently brought into focus as valid targets for anticancer therapies. These cells produce a variety of growth factors and extracellular matrix proteins in turn regulate the growth and progression of carcinoma cells.[20] Among several potential therapeutic targets of CAFs, fibroblast activation protein (FAP) which is specifically overexpressed by the fibroblast in tumor stroma, has long been considered a promising anticancer target molecule. Although humanized monoclonal anti-FAP antibody (Sibrotuzumab) did not result in disease remission in phase I/II clinical trial for colorectal cancer patient[21,22], targeting FAP merit further investigation due to recent data providing more evidences on the importance of stromal FAP in tumorigenesis.[23,24]

Another important group of cells to be targeted in the tumor microenvironment are the immune cells. Interactions between immune cells and tumor cells have been of great interest because of the possibility that they either restrict tumor progression or actively promote tumor growth. Recently, strategies aiming to manipulate the immune cells, particularly leukocytes such as regulatory T cells and pro-angiogenic/tolerogenic myeloid cells in the tumor microenvironment, have been emerging as potential therapy options. In this regard, immunomodulatory compounds like lenalidomide (Revlimid®) that modulate both cellular and humoral cells of the immune system and, other biologically important targets, have already been approved for the treatment of multiple myeloma and other similar lymphoproliferative diseases.[25]

To develop better therapeutic strategies targeted at the immune components of the tumor microenvironment, it is critical to understand how these cells are altered during tumor progression, and how they reciprocally influence tumor initiation and progression. Herein, the current understanding of tumor-stroma interaction is reviewed with a particular focus on tumor infiltrating leukocytes (TILs), which are the key component of the tumor stroma.[26] Moreover, the critical effects of targeting these crucial microenvironmental players on cancer vascularization, local tumor growth, metastatic spreading and anti-tumor immune responses are discussed in the following paragraphs.

2.1.2 Leukocytes in the tumor microenvironment

All tissues are comprised of diverse types of leukocytes, of both innate and adaptive lineages, as their normal cellular components. Solid tumors, mimicking the structure of normal tissue, also comprise various kinds and amounts of leukocytes depending on type and stage of the tumor (Figure 2.3). Leukocytes infiltrating tumors can constitute up to 50% of the tumor mass; the majority of these cells are myeloid cells (macrophages, neutrophils, dendritic cells, mast cells, eosinophils), as well as B and T lymphocytes.[27] Specifically, myeloid cells are the major component of the leukocytic infiltrates frequently seen in tumors.

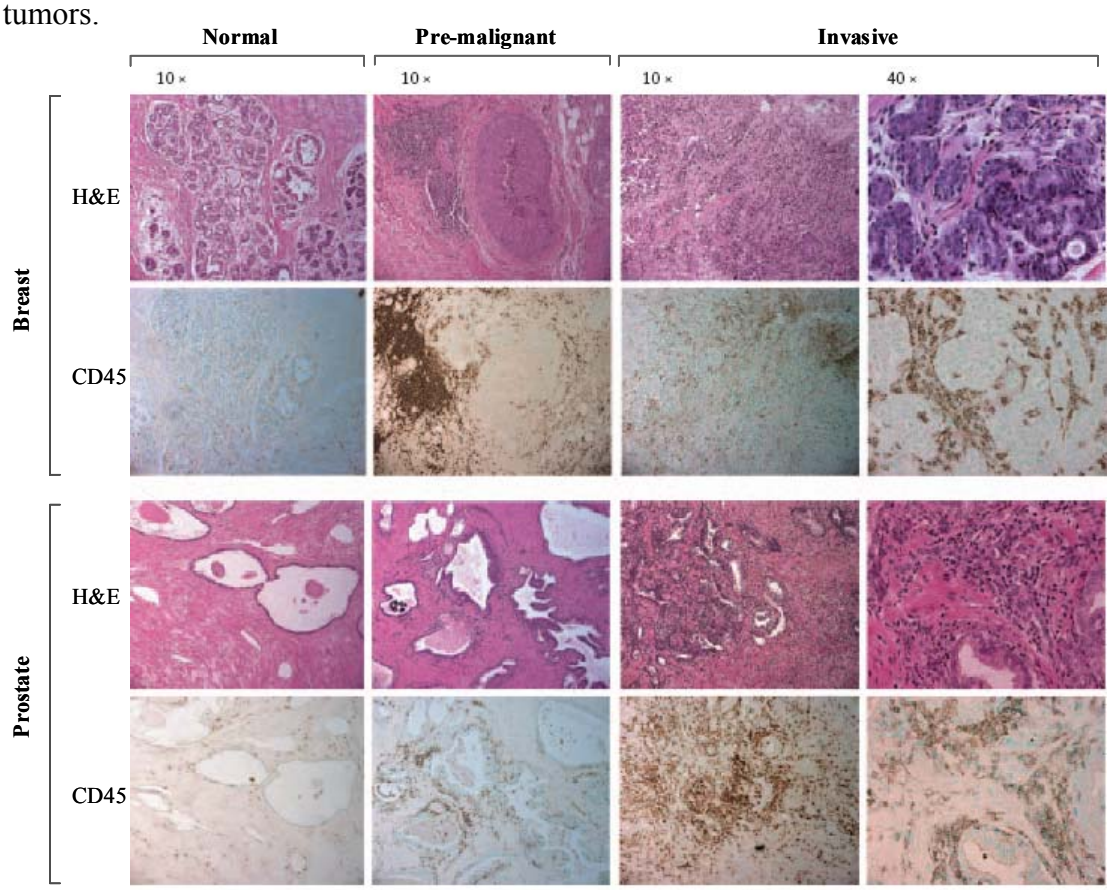


Figure 2.3: Representative sections of human breast and prostate carcinoma tissues depicting abundant leukocyte infiltration. Extensive leukocyte infiltration is a typical feature of many human cancers. Normal, pre-malignant and malignant tissue sections stained with haematoxylin and eosin (H&E) are shown (upper panels of each pair). Immunohistochemical staining for CD45, a pan-leucocyte marker, reveals the extent of leukocyte infiltration into pre-malignant and malignant stroma (lower, brown stained panels). Coussens et al., 2006.[28]

Infiltration of leukocytes into malignant tissue was first described by the distinguished pathologist Rudolf Virchow in 1863[29]; however, only recently we have begun to understand the diverse regulatory roles played by leukocytes in tumor growth. Originally, the presence of leukocytes in malignant neoplasms was thought to represent the host's immune response to a growing tumor. Yet, solid tumors are mostly recognized as "self" and do not evoke effective immune responses capable of eradicating tumors.[30,31] In contrast, extensive studies in experimental animal models revealed that these cells are actively recruited to neoplastic tissues by tumor cells and an abundance of certain types of leukocytes is associated with improved tumor progression. A large amount of clinical data derived from studies performed on a broad range of solid tumor types has confirmed the correlation between high-density leukocytic infiltration, and a poor outcome in patients.[32] Nevertheless in several cancers, the presence of certain types of leukocytes is associated with a favorable prognosis. For example, enhanced infiltration of natural-killer cells into tumors has been reported to correlate with a good prognosis in human ovarian, colorectal and gastric cancers.[33,34] Similarly, cytotoxic activation of lymphocytes, particularly $CD8^+$ T cells, in response to tumor growth result in tumor regression.[35] In sharp contrast, tumor-activated myeloid leukocytes were shown to restrain the protective function of these cells with anti-tumor activity, and subsequently promoted tumor growth and facilitated survival of neoplastic cells. Emerging data from clinical and experimental studies suggests that a high frequency of tumor-infiltrating lymphocytes are mostly associated with a good prognosis; whereas infiltration of myeloid cells correlates with a poor prognosis through increased angiogenesis, tissue remodeling, and suppression of anti-tumor immune response.[36-38] Since myeloid cells have been implicated in the regulation of these crucial aspects of tumor physiology, it is of paramount importance to understand how they are attracted to the tumor site, how they convey their tumor-promoting effects, and whether it is possible to block tumor-promoting activities while concurrently stimulating their anti-tumorigenic capacity.

2.1.2.1 Myeloid cell trafficking within tumor tissue

Myeloid progenitor cells reside predominantly in the bone marrow. In normal bone marrow, 50% to 60% of cells are dedicated to myeloid cell production. Under certain physiological circumstances, myeloid cells are recruited to the site of injury in response to wounding or inflammatory stimuli. Mobilization of myeloid cells in bone marrow, and recruitment to the site of damage, is mediated by local synthesis of chemokines, cytokines and growth factors. (see also 2.1.2.2.1, 2, 3) Throughout the wound-healing process, myeloid cells robustly promote healing by fighting infection, stimulating new tissue growth, followed by suppressing the response once the situation is resolved. In similar manner, these cells are actively recruited into malignant tissues by chemoattractants such as VEGF, PlGF, CCL2/MCP-1, CXCL12a/SDF-1α and β-defensins[39] and they also regulate similar processes to those observed during wound healing. However, due to accumulated mutations tumor cells lose the control of positional identity, and keep sending out continuous signals that recruit diverse myeloid cell populations to support tumor growth. This concept upholds the famous definition of tumors as "wounds that never heal".[29] Not only the tumor cells, but also the stromal cells mobilize various subpopulations of bone marrow-derived myeloid progenitor cells to the peripheral blood, and ultimately to malignant tissue through secretion of cytokines and chemokines (Figure 2.4).

Surface receptors on myeloid cells are regulated by these chemotactic factors and are involved in homing of myeloid cells to tumor tissue. (see also 2.1.2.2.1, 2, 3) This process is aided by increased endothelial cell adhesiveness for myeloid cells through enhanced expression of adhesion molecules.

Besides chemokines, concentration of oxygen in the tumor microenvironment also indirectly regulates homing of circulating myeloid cells to tissues. Hereof, hypoxia induced HIF-1 activity regulates the expression of certain chemoattractants, including MCP-1, CSF-1, VEGF-A, TNF-α, and SDF-1α.35 These chemoattractant factors do not only regulate the recruitment of myeloid cells, but also contribute to initial tumor-specific activation of these cells even before their arrival in the tumor. Once recruited into the tumor their function is further modulated by the tumor milieu.

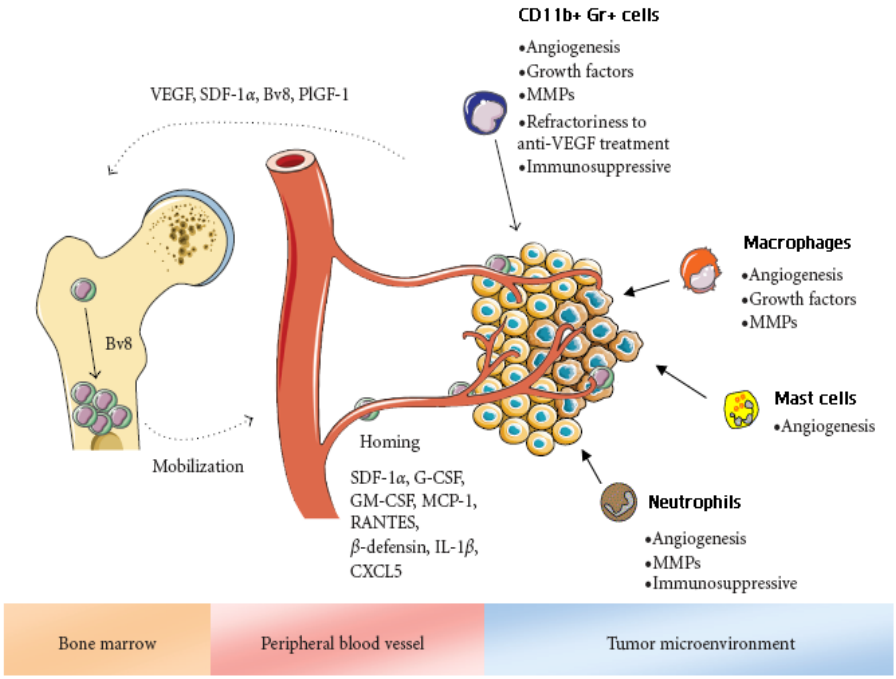


Figure 2.4: Recruitment of various myeloid linage cell types to the tumor microenvironment and their effects on tumor progression. Tumor and stromal cells mobilize diverse subsets of bone marrow-derived cells to the peripheral blood through secretion of cytokines and chemokines. Subsequently different chemoattractants promote the recruitment and infiltration of these cells to the tumor microenvironment where they suppress the anti-tumor immunity or promote tumor growth and angiogenesis. Schmid and Varner, 2010. [40]

2.1.2.2 Alternative activation of myeloid cells in the tumor microenvironment

It has become increasingly apparent that tumor progression is closely associated with abnormal expansion as well as impaired activation of myeloid cells, especially of the macrophages, neutrophils and dendritic cells along with the appearance of a heterogeneous set of immunosuppressive, immature myeloid cells called myeloid-derived suppressor cells (MDSCs). Myeloid cells could, in fact, induce an effective anti-tumor immune response by directly releasing cytotoxic factors and indirectly presenting antigens to lymphocytes or by releasing regulatory molecules to induce anti-tumor activity of T cells. They could also activate dendritic cells and natural-killer cells that can possess anti-tumor properties.[41] A number of *in vitro* studies have demonstrated the ability of macrophages to kill tumor cells

when appropriately stimulated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ).[42] Consistent with these properties, it was also shown in *in vivo* models that syngeneic macrophages originating from tumor-bearing mice inhibited melanoma growth in nude mice more efficiently than the control macrophages.[43] Although these findings suggest a possible involvement of myeloid cells in anti-tumor response, myeloid lineage cells instead drive tumor progression often by promoting cancer cell survival, angiogenesis, immunosuppression and metastasis. These effects can partly be explained by the absence of environmental stimuli inducing anti-tumorigenic polarization of myeloid cells at the tumor site. Instead, following recruitment they are likely to encounter factors that most frequently polarize them toward a pro-tumorigenic phenotype. This pro-tumorigenic activation of myeloid cells is determined by their interaction with cancer cells, as well as with other stromal compartments.

A variety of inflammatory stimuli can trigger the recruitment and polarization of diverse subsets of myeloid cells in tumor tissue. The following sections provide an overview of specific pro-tumorigenic myeloid populations, as well as their role in tumor progression and considering their crucial and well-documented tumor promoting properties, this text will particularly focus on macrophages, neutrophils and bone-marrow derived suppressor cells.

2.1.2.2.1 Macrophages

Macrophages are a major component of the myeloid infiltrate in a tumor microenvironment. Hence, of all cells of the myeloid lineage, they are among the most studied and evidenced for their contribution to tumor growth. Blood-circulating monocytes are recruited to tumors by tumor-derived chemotactic factors such as M-CSF (macrophage colony stimulating factor), CCL2 (chemokine C-C motif ligand 2, MCP-1) and VEGF (vascular endothelial growth factor). Upon migrating into the tumor, monocytes differentiate into tissue-resident macrophages called tumor-associated macrophages (TAMs). The term TAM defines intratumoral localization of macrophages. Macrophages are very plastic cells that can adapt a particular phenotype depending on the environmental stimuli, and they produce an assorted array of chemokines, cytokines, protesases,

angiogenic and growth factors. In response to diverse stimulants in the tumor microenvironment, they correspondingly undergo polarized activation. The activation states of macrophages, as well as of other myeloid cells, have been defined by a nomenclature adapted from the T_H1 and T_H2 cell response, referred to as M1 (classical) or M2 (alternative) activation, respectively. Classically activated macrophages (M1) are pro-inflammatory cells that are stimulated through exposure to factors such as IFN-γ or microbial products (like LPS) and possess a markedly enhanced ability to kill pathogens or tumor cells.[44] In contrast, when TAMs are more exposed to anti-inflammatory molecules, such as glucocorticoid hormones, IL-4, IL-13 and IL-10 they are polarized to the opposite extreme called M2. They are poor antigen presenting cells (APCs), which support tumor growth, angiogenesis, and metastasis, and they suppress the immune system by responding to anti-inflammatory cytokines, apoptotic cells and immune complexes. Differential cytokine production is a key feature of polarized macrophages. M1 macrophage activation in response to microbial products or IFN-γ is characterized by high levels of surface major histocompatibility complex class II (MHC-II) expression and antigen presenting capacity; high production of pro-inflammatory cytokines such as IL-12, IL-23, IL-1, TNF-α; high production of toxic intermediates (nitric oxide (NO)) and reactive oxygen intermediates (ROI).[45] In contrast, the M2 activation state is characterized by an IL-10^{high} and IL-12^{low} phenotype; expression of low levels of MHC-II and increased production of angiogenic factors and anti-inflammatory cytokines like IL-10, arginase and TGF-β. Furthermore, M1 macrophages express opsonic receptors (e.g., FcγRIII), whereas M2 macrophages express preferentially non-opsonic receptors (e.g., scavenger receptors like mannose receptors and CD163).[44] A comprehensive representation of the polarized functions of macrophages is shown in Figure 2.5.

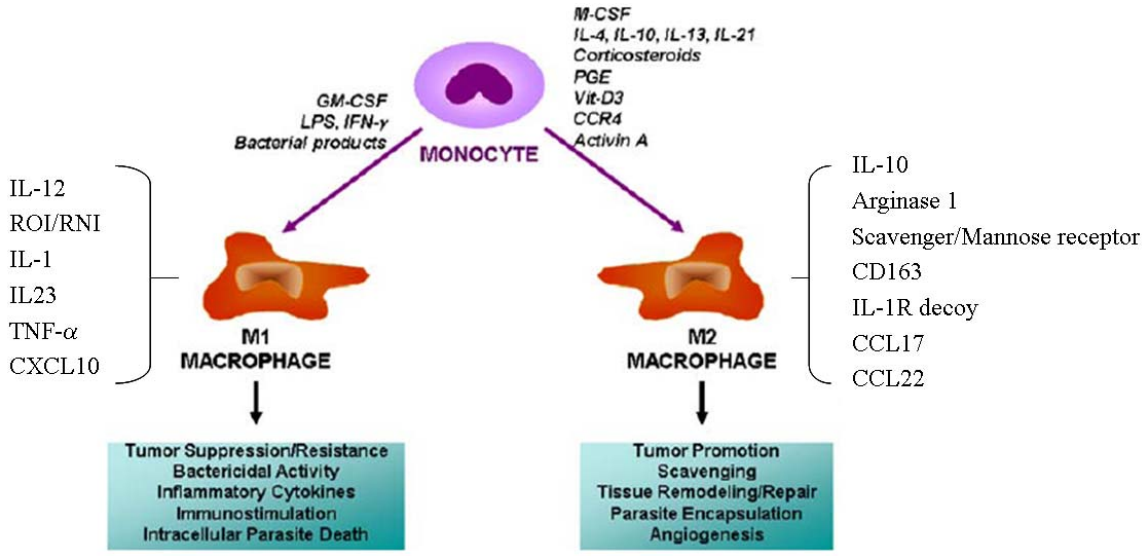


Figure 2.5: Polarization of macrophage function. Monocytes differentiate into polarized macrophages when exposed to diverse stimuli. In the presence of IFN-γ; granulocyte macrophage-colony stimulating factor (GM-CSF), LPS and other bacterial products, monocytes differentiate into M1-polarized cells eliciting microbicidal activity, immunostimulatory functions, and tumor cytotoxicity by producing TNF-*α*, IL-12, reactive nitrogen, and oxygen intermediates. On the other hand, when monocytes differentiate in the presence of IL-4, IL-13, IL-10 or corticosteroids, they mature into M2 macrophages that are involved in repair and remodeling of tissue, angiogenesis and tumor progression. Scholar et al.,2007 (modified).[46]

Due to the cytokine milieu of the tumor microenvironment, TAMs generally have a phenotype and function similar to M2 macrophages. They express high levels of M2 macrophage markers (IL-10, TGF-β, ARG1, CD163 and the mannose receptor) and low levels of mediators of M1 macrophage-mediated inflammation (IL-12, iNOS, TNF-α, and IL-6).[47] Still, this discrimination between M1 and M2 macrophages is rather a simplified view of these two extremes of polarization; it does not fully represent the continuum of functional states of macrophages in the tumor microenvironment.

Analysis of the molecular basis of the TAM phenotype identified components of the NF-κB signaling system as one of the main players in the modulation of macrophage function. Recently, Hagemann et al. revealed the requirement for IKKβ to maintain the M2 phenotype of TAMs in a mouse model of ovarian cancer.[48] Correspondingly, NF-κB inhibition by targeted deletion of IKK-β in TAMs increased their anti-tumor activity through reduced production of arginase-1, IL-10, TNF-α, as well as increased production of

inducible nitric oxide synthase (iNOS) and IL-12. These data suggest that IKKβ signaling in macrophages maintains their alternative tumor-promoting phenotype.[48] On the contrary, in tumors at a more advanced stage of progression, a therapeutic effect was achieved through the restoration of NF-κB activity in myeloid cells.[49,50] These divergent results may be associated with dynamic changes in a tumor microenvironment during transition from early neoplastic events towards advance stages. These changes may result in progressive modulation of NF-κB activity in tumor-infiltrating macrophages. Other important modulators of macrophage polarization are certain members of the STAT family of transcription factors. Although earlier evidence indicated that the STAT1 activation regulates the M1 activation of macrophages, recent reports argue against this, and suggest that STAT1 activation may induce TAM-mediated suppressive activity and tumor progression.[51-53] In addition, STAT3 and STAT6 activation were also shown to be associated with M2 macrophage polarization.[54,55]

2.1.2.2.2 Neutrophils

Neutrophils are short-lived white blood cells derived from bone marrow myeloid progenitors. During infection related immune responses, neutrophils are usually the first cells to arrive at the scene of infection, where they release chemokines and proteases that can, in turn, recruit both innate and adaptive immune effector cells. Neutrophils can also release several cytotoxic mediators, including reactive oxygen species, membrane-perforating agents, proteases and soluble mediators such as IFNs, TNF-α and IL-1β, suggesting their potential anti-tumor activity. Thus, they are considered as potential anti-tumor cells.[56] Though, this does not appear to be the case in cancer, and the role they play in tumors is still controversial. Low numbers of neutrophils are found in most of human tumors. Both cancer cells and stromal compartments of a tumor actively recruit neutrophils by means of secreted chemotactic factors, in particular G-CSF, GM-CSF, CXCL2/MIP-2α (functional homolog of human IL-8), CCL3/MIP-1α, CXCL5/LIX and CXCL1/KC. Upon being recruited to the tumor site, neutrophils can be tumor growth-stimulatory or -tolerant. An increased density of tumor-infiltrating neutrophils was found to correlate with a poor

prognosis in patients with adenocarcinoma and metastatic melanoma; whereas neutrophil infiltration in few cases like gastric carcinoma was linked to a good prognosis.[57-59] This discrepancy is probably related with the degree of recruitment and the differential activation of neutrophils, depending on the intratumoral cytokine microenvironment in which they act. Similar to TAMs, the functional status of tumor associated neutrophils (TANs) evidently governs their ability to express an anti-tumor potential. Accumulating experimental and clinical evidence also confirms that neutrophils can polarize in a type I or type II direction in tumors. Recently, Fridlender et al. characterized N1 and N2 polarized phenotypes of TANs, similar as described for TAMs. In lung and mesothelioma cancer models, TANs were shown to acquire a pro-tumor phenotype (N2). The pro-tumor activities of N2 TANs include the production of more immunosuppressive cytokines and reduced cytotoxic activity *in vitro*. This pro-tumor phenotype of neutrophils was found to be induced and maintained by TGF-β (Figure 6).[60] This finding is further supported by previous data indicating that TGF-β can inhibit neutrophil activity and cytotoxicity *in vitro*.[61] Also, a systemic blockade of TGF-β skewed the neutrophil differentiation toward the N1 phenotype that is characterized by the expression of more immunoactivating cytokines, lower levels of arginase and a higher capability of killing tumor cells *in vitro*. N1 polarized neutrophils execute anti-tumor activities indirectly as well, by promoting recruitment and activation of $CD8^+$ T cells (Figure 2.6). In addition to inducing the anti-tumor (N1) polarization, blocking of the TGF-β pathway led to increased recruitment of $Ly6G^+$ neutrophils in tumors.[60] This finding is consistent with previous studies demonstrating an enhanced influx of myeloid cells into mammary carcinomas deficient in type II TGF-β receptor.[62] In a parallel study, it was shown that abrogation of the TGF-β signaling in human breast cancer cells enhances the production of the neutrophil chemoattractants CXCL1 and CXCL5.[63] Apparently, TGF-β is one of the major players in regulating neutrophil recruitment and activation in the tumor microenvironment. A recent study by Weiss et al. suggested another factor that could influence neutrophil recruitment and differentiation in tumors. These authors reported that constitutive expression of IFN-β counteracts cancer-supportive function of neutrophils by inhibiting expression of genes encoding pro-angiogenic and homing factors in these cells. In this frame, IFN-β deficient

mice exhibited enhanced growth of melanoma accompanied by higher infiltration of neutrophils with pro-tumor properties.[64]

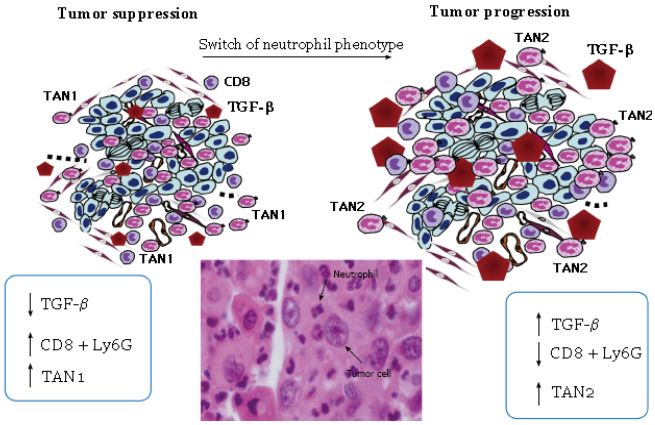


Figure 2.6: The proposed TGF-β mediated pro-tumorigenic polarization of neutrophils in the progression of tumors. Inhibition of TGF-β activity leads to the differentiation of neutrophils to anti-tumor N1 cells. While N2 cells inhibit the cytotoxic response of CD8 T cells, N1 cells enhance the anti-tumor action of $CD8^+$ T cells. The microphotograph depicts neutrophils (arrows) closely associated with carcinoma cells. Adapted from Hofman, 2010.[65]

Many other studies demonstrated that tumor-infiltrating neutrophils have an N2 phenotype that contributes to tumor progression and immunosupression.[64,66-68] Yet, when appropriately stimulated, these cells hold the potential to shift the tumor-leukocyte relationship toward tumor rejection.

2.1.2.2.3 Myeloid-Derived Suppressor Cells

Myeloid derived suppressor cells (MDSCs) are another well-described population of tumor-infiltrating myeloid cells that have been shown to exert a negative effect on the anti-tumor immune response. MDSCs are a heterogeneous population of cells comprised of monocyte, granulocyte, dendritic cell precursors and myeloid cells at an early stage of differentiation.[69,70] These cells are defined by the co-expression of monocytic marker CD11b and granulocyte differentiation antigen Gr1 (constituted by the two epitopes Ly6C and Ly6G). Recent studies broadly defined two major subsets of MDSC: cells of granulocytic ($CD11b^+Ly6G^+Ly6C^{low}$) and monocytic ($CD11b^+Ly6G^-Ly6C^{high}$) phenotype.[71,72]

It has been well established that the frequency of these cells significantly increases in the spleen and bone marrow of tumor-bearing mice, as well as in the peripheral blood and tumors of cancer patients. In naive tumor-free mice, MDSCs constitute ~30% of all bone marrow cells and ~3% of all nucleated splenocytes. However, they may represent

more than 20% of all splenocytes in tumor bearing mice.[73] In both patients and experimental animals, MDSCs have been shown to be mobilized from bone marrow in response to multiple tumor-derived factors such as Bv8 and endocrine gland-derived VEGF (EG-VEGF).[66,74] Their recruitment into the tumor site is then mediated by chemotactic factors like CCL2/MCP-1, CXCL12/SDF-1α, CXCL5 and KIT ligand.[75] Although they are able to differentiate into mature myeloid cells upon expose to appropriate stimuli, their differentiation is blocked under tumor cell conditioned media *in vitro* or in a tumor-bearing host *in vivo*.[76] These immature myeloid cells potently suppress maturation and anti-tumor activation of dendritic cells, T cells and natural killer cells, a phenotype that currently provides the most effective way of identifying MDSC.[77] Hence, injection of tumor cells in combination with $CD11b^+Gr1^+$ cells prompt tumor growth.[62] Accordingly, depletion of $Gr1^+$ cells in tumor-bearing mice leads to delayed tumor growth, suggesting MDSC as potential targets of anti-cancer therapy.[78]

Many reports have indicated that $CD11b^+Gr1^+$ cells from naive tumor-free mice are not immune suppressive.[72] However, it has not yet been uncovered why $CD11b^+Gr1^+$ cells derived from tumor-free and tumor-bearing animals exhibit different functions. Even in the same tumor-bearing mice, MDSCs isolated from the tumor site and from peripheral lymphoid organs display profound functional differences. In a recent study, Gabrilovich et al. suggested a HIF-1α mediated regulatory mechanism for the biological dichotomy displayed by MDSCs within the tumor microenvironment. These researchers demonstrated that MDSCs in the spleen of tumor bearing animals cause ROS mediated antigen-specific T cell unresponsiveness. On the other hand intratumoral MDSCs with similar morphology and phenotype suppress both antigen specific and nonspecific T cell function through an elevated level of NO and arginase I production. HIF-1α was found to be responsible for the altered functions of MDSC in the tumor microenvironment.[79]

2.1.2.3 Role of myeloid cells in tumor growth and metastatic spread

Due to their remarkable plasticity and capacity to produce a wide range of cytokines and chemokines, tumor-educated myeloid cells are the key regulators of tumor progression. Dense myeloid infiltration sites adjacent to areas of basement membrane breakdown and

tumor invasion are found in many tumor types.[36,80] Many other studies have documented a positive correlation between the number of myeloid cells and a poor prognosis.[28,39] Beyond that, genetic elimination and depletion of myeloid cells in animal tumor models were shown to impair tumor progression by altering various physiological processes that are necessary for tumor development.[81,82] These *s*tudies, as well as many others, have clearly demonstrated that many physiological processes that are critical to tumor development, such as cancer cell survival, angiogenesis, tissue remodeling, metastasis and immune suppression, are tightly regulated by myeloid infiltrates in the neoplastic environment.

2.1.2.3.1 Regulation of angiogenesis

As one of the hallmarks of cancer, angiogenesis is a very critical process for providing the necessary nutrients, oxygen and removal of waste products, as well as providing an escape route for metastasizing tumor cells.[83] Tumors at a size of 1-2 mm^3 alter their angiogenic phenotype to support continuous proliferation of endothelial cells.[83] Although various studies demonstrated that highly metastatic cell lines produce elevated levels of pro-angiogenic factors, tumor angiogenesis is likely initially stimulated by activated myeloid cells recruited into neoplastic tissue.[84]

Production of VEGF is one mechanism by which tumor infiltrating myeloid cells trigger and increase angiogenesis and foster tumor development. VEGF functions as a survival factor for endothelial cells, and induces angiogenesis by stimulating recruitment and proliferation of endothelial cells.[85] TAMs accumulate in poorly vascularized hypoxic or necrotic areas and respond to hypoxia by increasing the release of VEGF along with a number of other pro-angiogenic factors including TNF-α, fibroblast growth factor-2 (FGF-2), platelet derived growth factor (PDGF), urokinase-type plasminogen activator (uPA) and MMPs.[86,87] Hypoxic conditions in tumors stimulate expression of these pro-angiogenic molecules by activating hypoxia-inducible factors (HIFs) in macrophages. Activated macrophages also release nitric oxide (NO), a molecule that provokes increased vascular flow.[88] Lin and Pollard demonstrated that in the PyMT mouse model of mammary carcinogenesis, macrophages are recruited to pre-malignant tumors immediately before onset of the angiogenic switch that precedes the transition to a malignant phenotype.

Genetic ablation of macrophage infiltration into the neoplastic tissue delayed the angiogenic switch and tumor progression, whereas genetic restoration of the macrophage infiltration in these tumors rescued the vessel phenotype (Figure 2.7).[89]

Another myeloid population implicated in tumor angiogenesis are the MDSCs. Tumor-educated MDSC express elevated levels of the matrix degrading enzyme MMP-9 that triggers VEGF release from the extracellular matrix (ECM), which then induces proliferation of endothelial cells.[90,91] Interestingly, some tumors are resistant to anti-angiogenic blockage by VEGF antibodies.[92] A study by Shojaei et al. revealed that these refractory tumors were infiltrated with a higher frequency of $CD11b^{+}Gr1^{+}$ myeloid cells compared to sensitive tumors. These researchers further demonstrated that accumulation of $CD11b^{+}Gr1^{+}$ cells in tumors renders them refractory to anti-VEGF therapy, (most) likely because they are modulated by refractory tumor cells to produce alternative factors that can promote angiogenesis independently of VEGF. Finally, antibody mediated depletion of myeloid cells rendered the refractory tumors responsive to anti-VEGF therapy.[93]

Like other pro-angiogenic myeloid populations, tumor associated neutrophils have also been reported to support tumor growth by producing pro-angiogenic factors such as VEGF, IL-8 and certain proteases including MMPs and elastase.[94-96] Indeed, neutrophil-derived VEGF not only stimulates the migration and proliferation of endothelial cells, but also induces an elevated IL-8 secretion by endothelial cells, thereby enhancing further recruitment of VEGF-producing neutrophils.[97] This paracrine feed-forward mechanism was thought to enhance the angiogenic response by neutrophils. It is intriguing how neutrophils play a critical role in activating tumor angiogenesis in spite of their low abundance. For instance, neutrophils variably constitute 0.1–0.4 % of the total cells of solid tumors in a mouse model of pancreatic islet tumorigenesis (*RIP1-Tag2*). Nevertheless, systemic ablation of these cells during the early stage of tumor development significantly reduced the frequency of angiogenic switching.[98] These data support the hypothesis that the scarce neutrophils are the key initial source of MMP-9 that triggers the angiogenic switch through activation and release of latent VEGF. This significant effect of neutrophils might be partially explained with their critical localization at the interface between tumor and endothelial cells, as well as with their ability to produce crucial rate-limiting molecules.

The importance of myeloid cells in promoting tumor angiogenesis has been well investigated. Even so, the underlying molecular mechanisms remain to be fully explored. In this scope, Kujawski et al. reported that STAT3 activation in tumor-associated myeloid cells is critical for tumor angiogenesis. They identified that elevated STAT3 activity in TAMs and MDSCs up-regulated the expression of STAT3 pro-angiogenic target genes such as VEGF, MMPs and bFGF. Consequently, pro-angiogenic activity of myeloid cells was abolished when STAT3 was ablated in these cells. [99]

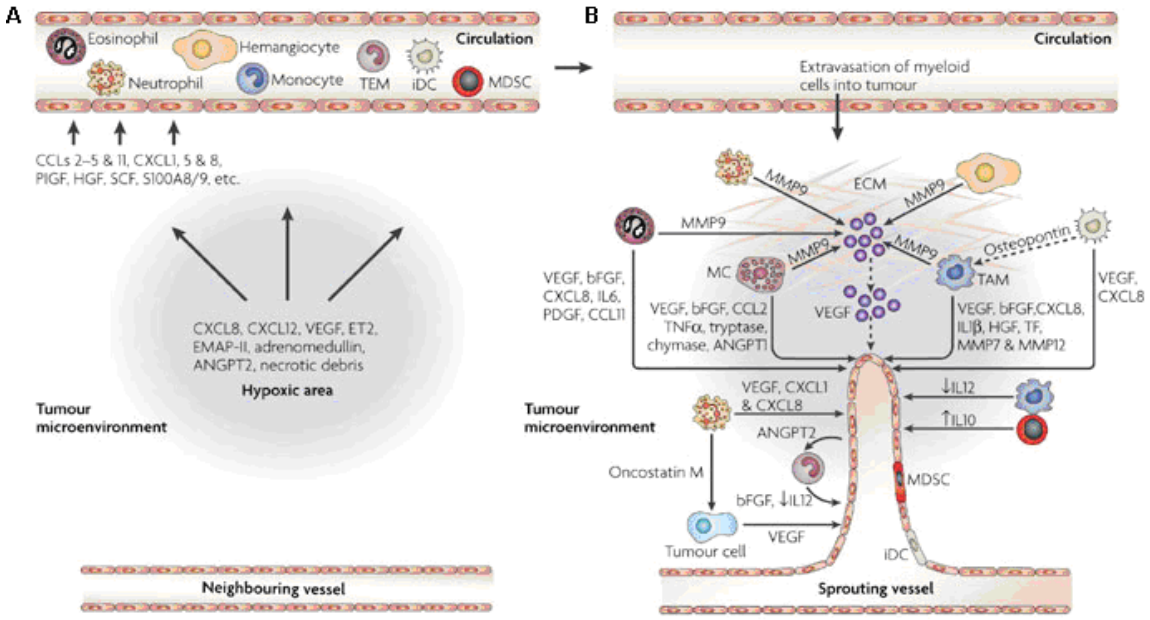


Figure 2.7: Myeloid cells promote angiogenesis in the tumor microenvironment. A.Malignant and stromal cell derived chemoattractants induce the infiltration of myeloid cells from the circulation into the tumor microenvironment. Some chemoattractants are also upregulated by cells in hypoxic and necrotic regions of tumor and may also attract certain types of myeloid cells into these regions. **B**.Following entry into tumor tissue, MDSC and TAMs accumulating in hypoxic sites release pro-angiogenic factors. These cells, along with neutrophils throughout the tumor also secrete MMP-9 and bio-active VEGF, which then induce angiogenesis. Moreover, contact of TAMs with MDSC causes TAMs to downregulate the anti-angiogenic factor IL-12 and MDSC to upregulate IL-10. A subset of macrophages, the TIE2-expressing monocytes (TEM) secrete angiogenic factors. MDSC were shown to be capable of trans-differentiating into endothelial-like cells *in vitro* and that they may become incorporated into new blood vessels in tumors. Adapted from Murdoch et al., 2008.[100]

2.1.2.3.2 Regulation of tumor growth and survival

Beyond activating vascularization, tumor-infiltrating myeloid cells can also promote neoplastic growth by creating a microenvironment that is rich in growth factors and cytokines that can stimulate proliferation and survival of neoplastic cells.[101] Growth-promoting effects of various myeloid cell-derived cytokines were shown in different tumor models like IL-6 in hepatocellular carcinoma,[102,103] TNF-α[104] and IL-6[105] in colitis associated cancers. Other growth factors specifically secreted by TAMs have also been implicated in tumor growth: epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF).[75,106] Accumulating data for solid tumors conveyed a correlation between TAM infiltration and tumor cell proliferation.[107,108]

Besides directly promoting tumor cell proliferation, tumor-educated myeloid cells can also indirectly facilitate tumor growth through suppression of anti-tumor immune responses, allowing the tumor escape from immune surveillance. Different mechanisms have been suggested for the immunosuppressive effects of myeloid cells, such as secretion of immunosuppressive cytokines, generation of reactive oxygen species (ROSs) and increased activity of arginase and nitric oxide (NO). MDSCs are thought to play a major role in preventing the immune system from attacking tumor cells. In recent years, they have been shown to suppress the immune system through multiple mechanisms, including inhibition of T cell activation by direct cell to cell contact, by production of immuno-suppressive mediators, and also by inducing regulatory T cells (Treg).[73,109,110] One important immunosuppressive mediator that is highly produced by the MDSC is arginase, which causes a reduction in the arginine necessary for T cell activation.[111] Another MDSC-derived immunosuppressive mediator, TGF-β, also converts naive $CD4^+$ T cells into adaptive Tregs.[112]

Normally, macrophages from healthy individuals bear the potential to lyse tumor cells and present tumor-associated antigens and thereby activate the anti-tumor response of T cells and NK cells. In contrast, tumor-educated macrophages act oppositely and inhibit anti-tumor response through several mechanisms. Initially, they are limited in their ability

to present tumor associated antigens to T cells due to the presence of tumor derived factors such as IL-10, TGF-β1 and PGE_2 suppressing the expression of MHC class II molecules. In turn, they themselves produce IL-10, TGF-β and PGE_2 that contribute to suppression of the cytotoxic T cell response.[113,114] Not only the intratumoral macrophages, but also spleen and peritoneal macrophages of tumor-bearing individuals share these similar immunosuppressive properties.[115] Next, TAMs were also shown to foster a tumor's immune privilege by attracting $CD4^+CD25^+FOXP3^+$ Tregs that are known to suppress the anti-tumor function of cytotoxic T cells. Accumulation of Tregs in tumors is a common feature of human cancers and the abundance, as well as suppressor activities of these cells, were found to be highly correlated with a poor prognosis.[116] Using ovarian carcinoma samples from human patients, Curiel at al. revealed that TAMs regulate Treg trafficking to tumors by producing CCL22, a chemokine that mediates regulatory T cell recruitment.[117]

The pioneering work of Fridlender et al. demonstrated that N2 polarized neutrophils control the activation status of CD8 cells in the tumor microenvironment. Comparatively, tumor infiltrating CD8 cells can also control the activation and migration of tumor-associated neutrophils.[60,118] Finally, the release of reactive oxygen species, i.e., H_2O_2, during the oxidative burst of tumor-activated neutrophils has been suggested to be the underlying mechanism of systemic T-cell dysfunction in advanced cancer patients.[68]

2.1.2.3.3 Regulation of invasion and metastasis

Originally, the ability of a tumor to undergo invasion and to metastasize in a specific organ was thought to be highly cancer cell specific. However, recent findings have challenged this notion. Myeloid cells were found to be critically contributing to those steps of tumor progression by producing several factors which enhance tumor cell invasion and metastasis. One of the main mechanisms by which these cells support tumor metastasis is the production of enzymes such as MMPs, plasmin and urokinase type plasminogen activator (uPA) which regulate the digestion of extracellular matrix, thus favoring tumor cell invasion. Direct evidence has shown that MMP-9 supplied by myeloid subsets like macrophages, neutrophils and mast cells, increase the incidence of invasive epidermis

cancer in a mouse model of multi-stage tumorigenesis.[119] The critical role of myeloid cells in tumor metastasis was also confirmed by mouse tumor models in which myeloid cells were either genetically or pharmaceutically ablated. In a mouse mammary cancer model, Lin et al. reported that the absence of CSF-1, a macrophage growth factor, significantly blocked intratumoral infiltration of macrophages, and consequently delayed both the tumor progression to malignancy and metastatic spread to the lung. Furthermore, restoration of the expression of CSF-1, specifically in the mammary epithelium of null mutant mice, reverses the phenotype and accelerates both tumor progression and metastasis.[81] Neutrophils in tumor microenvironments were also reported to be critical for the acquisition of a metastatic phenotype. In a murine fibrosarcoma model, they have been shown to promote the poorly tumorigenic tumor cells to acquire an aggressive metastatic phenotype. Genetic and chemical ablation of neutrophil infiltration dramatically suppressed the metastatic phenotype of tumors as compared with those in control mice.[67]

Recent evidence suggests another interesting concept: Primary tumors are able to modify the distant sites prior to the arrival of metastatic cells to create a permissive microenvironment, a so-called "pre-metastatic niche".[120] Bone marrow derived myeloid cells are thought to be major executers of this process.[121,122] In a pioneering study which attempted to investigate the molecular mechanism of tissue-specific metastasis, Hiratsuka and colleagues showed that MMP-9 is specifically induced in pre-metastatic lung endothelial cells and macrophages by distant primary tumors, suggesting that it significantly promotes lung metastasis.[123] A later report by Kaplan et al. supported those findings and further showed that $VEGFR1^+$ bone marrow-derived hematopoietic progenitor cells home to tumor-specific pre-metastatic sites and establish cellular clusters before the arrival of tumor cells.[121] These clusters alter the microenvironment by MMP-9 production and enhanced expression of SDF-1 creating a chemokine gradient that permits the attraction of tumor cells and their incorporation into the niche. Afterwards, Hiratsuka et al. further reported that primary tumor-mediated attraction of Mac 1^+ (macrophage antigen 1)-myeloid cells to the pre-metastatic lungs induces the colonization of the lung by tumor cells. Secretion of certain factors like, TGF-β, TNF-α, and VEGF-A by the primary tumor

stimulates expression of the inflammatory chemoattractants S100A8 and S100A9, which in turn attract $CD11b^+$ ($Mac\ 1^+$) myeloid cells to the pre-metastatic milieu.[122]

In short, all these studies provided evidence that the different myeloid subsets alter the pre-metastatic lung environment, making it easier for tumor cells to extravasate from blood vessels and to colonize the lung.

2.1.3 Modulation of myeloid cells in the tumor microenvironment: a new prospect in cancer therapy

Based on a vast amount of clinical and pre-clinical evidence, current knowledge clearly suggest that therapeutic targeting not only of the cancer cells, but also of the microenvironment is necessary for an effective inhibition of tumor growth. As a result, interference with the microenvironmental growth support is becoming more widely appreciated as an attractive therapeutic strategy. As a key component of the tumor microenvironment, tumor promoting properties of myeloid cells define these cells as valuable targets for therapeutic interventions. To this end, depletion of myeloid cells may increase the therapeutic benefit, and perhaps prevent metastatic progression, but it also results in prolonged suppression of innate immunity. Since they are critical mediators of host defense against infections, depletion of myeloid cells may not be feasible in human patients. Furthermore, the same myeloid cells have the potential to elicit an anti-tumor immune response, and suppress the malignant progression, if appropriately stimulated. However, attempts to stimulate the immune system to mount an effective anti-tumor response mostly aimed to utilize T-cell based strategies. Although the significance of MHC class I-restricted cytotoxic T lymphocytes (CTLs) as effectors of anti-tumor immunity has been widely demonstrated, most human tumors lack MHC class I expression, or are so inadequately differentiated and poorly immunogenic that success of T-cell based tumor-specific immunotherapy is rather limited.[42,124-126] Effective host anti-tumor reactivity could better be implemented by cells of innate immunity, such as macrophages, NK cells, and granulocytes. Difficulties raised by the recognition of specific tumor-associated antigens, and due to absent and defective MHC-class I antigen expression, are thus evaded.

With this in mind, it might be possible to develop novel strategies aimed at re-programming tumor associated myeloid cells in favor of an anti-tumor phenotype. Based on the M1 versus M2 paradigm of macrophage polarization, inhibition of M2 and activation of M1-inducing signals was suggested as a potential strategy to re-establish the anti-tumor function of macrophages.[127] Indeed, accumulating evidence has indicated that pharmacological skewing of macrophage polarization, from M2 to M1 phenotype, is able to maintain an anti-tumor activity. For example, in a study with different tumor models, Guiducci et al. tested the hypothesis whether the treatment of tumor-bearing mice with a macrophage chemoattractant would synergize with a treatment that would simultaneously switch the macrophage phenotype from M2 to M1. Administration of macrophage chemoattractant CCL16, in combination with a microbial stimulus (CpG oligonucleotide, a Toll-like receptor-9 ligand), and an anti-IL-10 receptor antibody was shown to promptly skew the tumor-infiltrating macrophage phenotype from M2 to M1 that triggered an innate response regressing pre-established large tumors.[128] Considering their central role in the polarization of myeloid cell functions, members of the STAT family of transcription factors are valuable targets for the modulation of myeloid cells. Activation or inactivation of specific STATs is, therefore, a potential strategy to restore the anti-tumoral function of myeloid cells. A representative example is provided by the work of Rosenberg et al. wherein TAMs from mammary tumor bearing $STAT6^{-/-}$ mice were shown to display an M1 phenotype, characterized with low level expression of ARG1 and high level expression of NOS2.[129] $STAT6^{-/-}$ mice with M1 macrophages were also shown to reject spontaneous 4T1 mammary carcinoma.[130] In the same line, Rauh and colleagues demonstrated the critical role of another factor, SHIP1 phosphatase, in programming macrophage M1 versus M2 functions. Mice deficient in SHIP1 displayed a skewed development toward M2 macrophages, and thus pharmacological modulators of this phosphatase are under investigation.[131] More recently, a host-derived factor, histidine-rich glycoprotein (HRG) was reported to promote M1 polarization of TAMs. It was shown that HRG induced M1 macrophage-mediated anti-tumor immune response and vessel normalization that subsequently inhibited tumor growth and metastasis, while improving chemotherapy.[132]

To date, the anti-tumor potential of neutrophils has received little attention, and scientists have not yet fully considered the possibility of utilizing their functions as effective weapons against cancer. Yet, compelling evidence recently gathered signifies that under appropriate stimulation neutrophils reveal very powerful tumor-inhibitory properties. The functional status of a granulocyte evidently governs its ability to express its anti-tumor potential. As neutrophils in tumor-bearing individuals have impaired cytotoxic activity, the elaboration of systems capable of stimulating the recruitment of neutrophils, and their anti-tumorigenic activation within a tumor microenvironment can be suggested as a new therapeutic approach. Such an approach has been tested in a number of settings. Early studies with mammary adenocarcinoma cells transfected with cytokine and chemokine genes, and injected into syngeneic mice, indicated that nonspecific mechanisms, mostly supported by neutrophil function, have much greater therapeutic power than those elicited by specific immunity.[133-135] In particular, local or systemic administration of rIL-12 in mice bearing subcutaneous mammary carcinoma resulted in a rapid influx of neutrophils with high destructive potential and anti-angiogenic function.[136] Finally, TGF-β has been defined as a major regulator of neutrophil's functional status. Specifically in tumors, TGF-β has been noted to drive pro-tumorigenic polarization of neutrophils. Along these lines, inhibition of TGF-β signaling offers a means to manipulate neutrophil polarization *in vivo* that can shift the tumor/neutrophil relationship toward tumor inhibition. As mentioned in section 2.1.2.2.2**,** TGF-β receptor blockage in tumor bearing mice was shown to induce the activation of $CD11b^{+}Ly6G^{+}$ neutrophils with an anti-tumorigenic phenotype that resulted in a significant tumor growth delay.[60]

Together, all these findings indicate the significance of re-programming myeloid cell phenotypes to affect tumor outcome and, accordingly, suggest it as a promising strategy to complement the established anticancer treatments. The major question that arises with regard to this capability then becomes what are the potential endogenous or environmental factors that could modulate the state of activation of these myeloid cells and what are the cellular and molecular mechanisms through which these factors regulate the modulation?

In this context, the following section provides an overview of bisphosphonates, a class of drugs that might have potential immune-modulatory properties on innate cells in cancer. Their pharmacological properties and documented anti-tumor activities are given with a special emphasis on zoledronate that is a new generation bisphosphonate and the focus of this study.

2.2 Bisphosphonates

2.2.1 General properties of bisphosphonates

Bisphosphonates (BPs) are inorganic pyrophosphate analogs (PPi) that effectively inhibit osteoclastic bone resorption and, accordingly, are widely used in medicine to treat metabolic bone diseases, such as postmenopausal osteoporosis,[137] Paget's disease,[138] tumor associated osteolysis,[139] and more recently to prevent bone metastasis. [140] The high affinity of the bisphosphonates for the calcium component of the bone matrix (hydroxyapatite) is the origin of the bone-specificity of these compounds. *In vivo* organ distribution studies demonstrated that bisphosphonates are mainly localized in newly formed bones and subsequently internalized by cells involved in bone resorption, namely osteoclasts where they inhibit their activity.[141] Because osteoclasts are highly endocytic cells, bisphosphonates released from degraded bone matrix are likely to be taken up by osteoclasts through endocytosis, thereby affecting them directly. Nevertheless, this does not exclude the possibility that small amounts of these drugs are internalized by adjacent cells (such as osteoblasts, bone marrow cells, or tumor cells in case of bone tumors). Due to their high affinity for bone matrix, systemic availability of bisphosphonates is rather low with the exception of a transient raise of plasma levels in the post-administration period.[142]

Based on their chemical structure bisphosphonates can be divided into two distinct pharmacological classes; nitrogen-containing bisphosphonates, (N-BPs, e.g. zoledronate) and non-nitrogen containing bisphosphonates (non-N-BPs, e.g. clodronate), which more closely resemble PPi. Pyrophosphate has a P–O–P structure, two phosphate groups linked by an oxygen atom. Bisphosphonates have a P–C–P structure, where the central oxygen atom is replaced by a carbon atom. As shown in Figure 2.8, the first generation bisphosphonate, clodronate has R^1 and R^2 side chains with two chlorine atoms attached to

the central carbon atom, whereas the new generation bisphosphonate zoledronate (1-Hydroxy-2-(imidazol-1-yl-amino) ethylidene-1,1-bisphosphonic acid) has a R1 side-chain with a hydroxyl group and an R2 side-chain with a imidazolyl group, that strongly influence bone binding and anti-resorptive properties. Bisphosphonates containing nitrogen atoms in the R^2 side-chain like zoledronate are more potent than non-nitrogen bisphosphonates.[142]

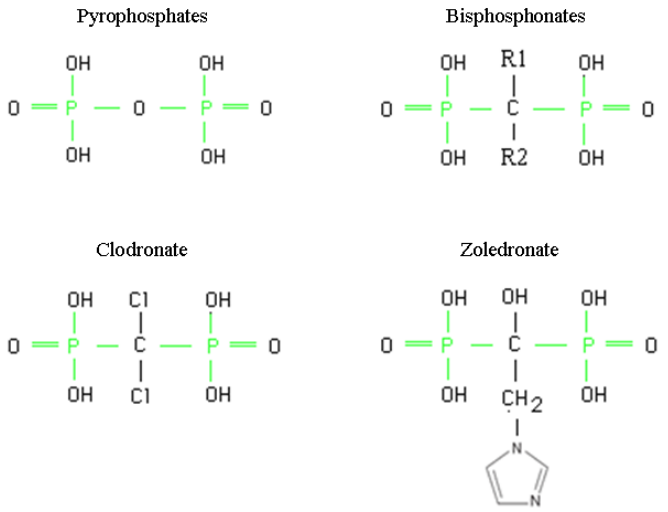


Figure 2.8: The chemical structure of pyrophosphates, generic bisphosphonates, clodronate and zoledronate. Pyrophosphate has two phosphate groups linked by an oxygen atom. Zoledronate contains a tertiary amine within an imidazole ring, making zoledronate one of the most potent available bisphosphonate to date.

2.2.2 Molecular mechanism of bisphosphonate activity

The molecular mechanism of the bisphosphonates differs according to their chemical structure. After cellular uptake, non-nitrogen-containing bisphosphonates (e.g., clodronate) are metabolized to cytotoxic analogs of adenosine triphosphate (ATP). Accumulation of cytotoxic ATP analogs in the cell cytoplasm directly causes apoptosis by inhibiting mitochondrial adenine nucleotide translocase (ANT).[143] On the other side, the more potent nitrogen-containing bisphosphonates exert their effects mainly by inhibiting a key enzyme in the mevalonate pathway, farnesyl pyrophosphate synthase (FPP synthase, Figure 2.9), thereby preventing the synthesis of isoprenoid compounds that are essential for the posttranslational modification (e.g. prenylation) of small guanosine triphosphate (GTP)-binding proteins (small GTPases) such as Rab, Rho, and

Rac. Small GTPases are significant signaling proteins that regulate various cellular processes critical for cell function, including cell morphology, cytoskeletal arrangement, trafficking of vesicles, and apoptosis. Prenylation is required for the proper functioning of these proteins because the lipophilic prenyl group serves to anchor the proteins in cell membranes. Therefore, impaired prenylation of small signaling proteins may cause loss of cellular functions that may be followed by apoptotic cell death.[144] Moreover, previously it has been shown that N-BPs are not metabolized into ATP analogs. However, recent studies revealed that N-BPs can also induce formation of a new pro-apoptotic ATP analog (ApppI, Figure 2.9), as a consequence of the inhibition of FPP synthase in the mevalonate pathway. Similar to the ATP analog of non-N-BPs, ApppI can induce mitochondria-mediated apoptosis.[145]

The differences at the BP side chain produce significant diversity in the potency of various bisphosphonates. Among those tested *in vitro*, zoledronate has the highest potency of inhibiting the human farnesyl diphosphate synthase.[146] In preclinical models of bone resorption, the newer bisphosphonates, such as ibandronate and zoledronate, show 10.000- to 100.000-fold higher potency than the older agents such as etidronate and clodronate.[147] Due to its high potency and efficacy in treatment of bone metastasis, recent interest has focused on the use of zoledronate in cancer.

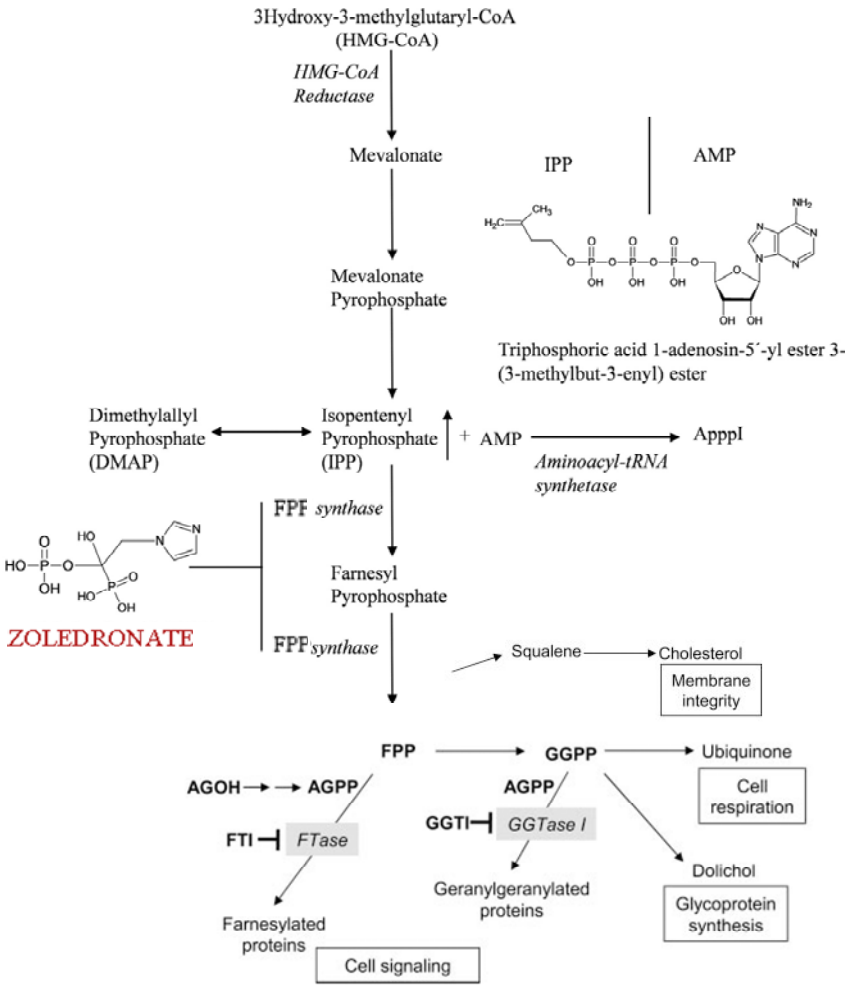


Figure 2.9: The mevalonate pathway and proposed mechanism of action for zoledronate. Inhibition of farnesyl diphosphate synthase by zoledronate disrupts isoprenoid biosynthesis. Insufficient levels of isoprenoid species (farnesyl pyrophosphate, FPP, and geranylgeranyl pyrophosphate, GGPP) cause numerous cellular effects, including loss of membrane integrity due to deficient cholesterol synthesis, altered cell signaling by blockade of post-translational modification of many proteins. In addition, inhibition of FFP synthase leads to accumulation of isopentenyl pyrophosphate (IPP). IPP is conjugated to AMP to form a novel ATP analogue, ApppI. Adapted from Mitrofan et al., 2010.[148]

2.2.3 Pharmacokinetics and biodistrubition of zoledronate

Since it is a highly charged and water soluble compound, zoledronate robustly chelates divalent cations like calcium and thus readily binds to bone.[149] *In vivo* studies demonstrated that up to one-half of the administered zoledronate is directly eliminated from the body by the kidneys, without biotransformation. The rest is located to bone,

where it accumulates. Bone acts as a reservoir for zoledronate and osteoclast mediated bone resorption gradually releases the drug during normal bone remodeling. In general, zoledronate is a well tolerated drug. The most common adverse effects include skeletal pain, anorexia, constipation, fever and nausea.[150] Osteonecrosis of the jaw bone is a less common side effect that has been reported mainly in patients with cancer who are undergoing dental surgery.[151]

Animal studies revealed that concentrations of zoledronate in soft tissue were about 1–3 orders of magnitude lower than in bone.[152] Interestingly, encapsulating bisphosphonates into liposomes to target them for macrophage phagocytosis modifies their selectivity from osteoclasts to macrophages, and *i.v.* or *i.p.* application of liposomes containing clodronate can deplete macrophages in the mononuclear phagocytic system (MPS), formerly called reticuloendothelial system (RES).[153]

2.2.4 Anti-tumorigenic properties of zoledronate

Although zoledronate has originally been developed to inhibit osteoclast mediated bone resorption, ongoing studies are now evaluating the anti-cancer effects of this compound. In the cancer context, zoledronate is primarily used as adjuvant therapy to inhibit local bone destruction by tumors and to prevent or delay metastases to the bone. Bone metastases are common in patients with different types of cancers; particularly lung, breast and prostate cancers are associated with severe skeletal complications including bone pain, pathological fractures, spinal cord compression and hypercalcemia, all of which substantially reduce the patient's quality of life. These skeletal morbidities can be treated to a certain extent by exploiting the bone resorption inhibitory properties of zoledronate.

In this regard, a panel of bisphosphonates (including clodronate, pamidronate, zoledronate, ibandronate) was clinically tested in advanced breast cancer patients with bone metastases. Among the tested BPs, zoledronate exhibited the most substantial and consistent clinical benefits related to skeletal morbidity.[154,155,156] In another clinical trial with advanced renal cancer patients, multiple event analyses have demonstrated that zoledronate reduced the risk of developing skeletal related events (SRE) by 61% as

compared with placebo.[157] Moreover, zoledronate has demonstrated significant clinical benefits in patients with metastatic prostate and lung cancer.[157,155] In the latter, zoledronate was shown to delay the median time to first SRE (236 days with ZA vs. 155 days with placebo) and to significantly reduce the annual incidence of SREs (1.74 per year with ZA vs. 2.71 per year with placebo). Eventually, the drug was found to reduce the risk of developing a skeletal event by 31%. Based on these findings, phase III clinical trials in cancer patients were completed and zoledronate is registered in the United States and Europe for the treatment of bone metastases associated with a wide variety of tumors.[158]

Numerous recent publications indicated that zoledronate does more than just inhibit osteoclast function in the bone. Besides preventing cancer related skeletal morbidities, it exhibits diverse anti-tumor activities that can be classified as direct and indirect effects. Zoledronate exerts these anti-tumorigenic activities directly on cancer cells by modulating their tumorigenic properties and indirectly on stromal cells by modulating their tumor-promoting properties.[159]

2.2.4.1 Direct effects: Modulation of cancer-cell properties

2.2.4.1.1 Anti-proliferative and pro-apoptotic effect

Direct effects of zoledronate on cancer cells are mainly tested in *in vitro* settings. One of the major anti-tumor effects of zoledronate is the induction of apoptosis. Early studies have demonstrated an increase in the proportion of cells with altered nuclear morphology and fragmented DNA, characteristic of apoptosis, in zoledronate treated myeloid cell lines.[160] Besides studies exhibiting pro-apoptotic effects, there are various reports describing *in vitro* anti-proliferative effects of zoledronate on different types of cancer cells. In a study with the myeloma JJN-3 cell line, the compound was shown to arrest the cells in the S-phase of the cell cycle.[161] Furthermore, dose and time-dependent anti-proliferative and pro-apoptotic effects of zoledronate were also shown in breast cancer, prostate cancer, pancreatic cancer, lung cancer and osteosarcoma cells *in vitro*.[162-164] Several studies attempted to elucidate the mechanisms of the pro-apoptotic effect suggested that inhibition of small GTPase prenylation in the mevalonate pathway might

be the main mechanism driving zoledronate mediated tumor cell apoptosis.[165,166] A study with MCF-7 breast cancer cells supported this notion by demonstrating that apoptotic activities of zoledronate could be reversed by incubation with geranylgeraniol, a downstream metabolite of the mevalonate pathway.[162]

Although *in vitro* data suggest a promising pro-apoptotic effect of the drug on tumor cells, the question if this cytotoxic potential translates into equivalent *in vivo* effects in tumor models is currently under investigation. Zoledronate was found to induce apoptosis in breast cancer cells at a concentration range between 10-100 μM *in vitro*.[167] A critical question is whether these cytotoxic concentrations can be reached in animal tumor models and consequently in human patients. As mentioned before, systemic concentration of zoledronate is considerably low. Following *i.v.* administration of a standard 4 mg dose of zoledronate, the drug remains in the plasma for 1–2 h before locating to bone. The peak plasma levels are estimated to be 1–2 μmol/l that is critically lower than the concentration necessary to exert direct anti-tumor effects.[168,169] However, local drug concentrations in the bone microenvironment may be sufficient to inhibit growth of tumor cells in this microenvironment. For this reason *in vivo* studies have mainly focused on the effect of the drug on tumors growing in bone microenvironment like multiple myeloma or on bone metastases of various cancers. In this context, *in vivo* data have been published showing that zoledronate may have the potential to reduce the tumor load and induce tumor cell apoptosis in corresponding animal models. [170,171]

2.2.4.1.2 Inhibition of adhesion, migration and invasion

Zoledronate not only influences proliferative and apoptotic properties of cancer cells, but also interferes with migratory and invasive properties. Based on results from matrigel-based invasion assays, zoledronate appears to inhibit the potential of human prostate and breast cancer cells to adhere and invade into the extracellular matrix.[172] These activities could have further implications on the metastatic potential of these cancer cells. In MCF-7 and MDA-MB-231 breast cancer cells, low concentrations of zoledronate (100 nM - 10 μM) reduced the number of cells adhering to mineralized and non-mineralized matrices by 40-80 %.[173] The anti-adhesion effect could not be due to

cytotoxicity, because zoledronate is not cytotoxic at doses capable of inhibiting adhesion. Since the peak plasma concentration of bisphosphonates in humans is in the micromolar range, *in vitro* anti-adhesive and anti-invasive effects of zoledronate observed at sub-micromolar concentrations could also be applicable *in vivo*. In an attempt to uncover the molecular mechanisms mediating the anti-invasive activities of zoledronate, Teronen et al. demonstrated that the matrix metalloproteinases MMP-3, -12, -13, and -20 are inhibited by zoledronate in osteosarcoma, melanoma and fibrosarcoma cell lines *in vitro*.[174] Another study by Boissier and colleagues suggested that zoledronate did not interfere with the production of MMPs by tumor cells, but inhibited their proteolytic activity through zinc chelation.[175]

2.2.4.2 Indirect effects: Modulation of tumor microenvironment

Apparently, zoledronate can directly affect certain properties of tumor cells, albeit mostly within the bone microenvironment. Since, as mentioned before, serum concentrations of zoledronate are far below the required threshold to directly inhibit tumor cell proliferation or induce apoptosis, it is not clear whether its anti-tumor activities could reach beyond the bone. Moreover, physicochemical properties like negative charges of zoledronate prevent it from moving across cell membranes and minimize cellular uptake. In this respect, potential anti-tumor effects of zoledronate in non-skeletal tumors cannot be solely attributed to direct killing of cancer cells. A few recent studies demonstrated the anti-tumor activities of zoledronate on soft-tissue tumors.[176-178] These findings suggest that clinically observed effects of zoledronate treatment may also be explained by indirect mechanisms regulating the modulation of the tumor microenvironment.

2.2.4.2.1 Inhibition of angiogenesis

Zoledronate was shown to impair tumor growth indirectly by inhibiting angiogenesis. Anti-angiogenic properties of the compound were evaluated in a number of studies. *In vitro* assays showed that treatment of human umbilical vein endothelial cells with low concentrations of zoledronate (1-30 μM) inhibited cell proliferation, whereas

higher concentrations (100 μM) induced apoptosis.[179] In another study, zoledronate was also shown to inhibit differentiation and the ability to form capillary-like tubes of endothelial progenitor cells even at low concentrations (1 μM).[180]

Results of several *in vivo* studies demonstrated that zoledronate inhibits angiogenesis in experimental angiogenesis models and lowers the levels of circulating pro-angiogenic factors in tumor bearing mice and cancer patients.[181] The first study indicating an inhibitory effect of zoledronate on angiogenesis occurring in non-mineralized tissue was performed by Wood et al.[179] In this study, systemically administrated zoledronate inhibited angiogenesis induced by subcutaneous implants impregnated with bFGF and VEGF in a mouse model. In a transgenic mouse model of cervical cancer, zoledronate therapy (100 μg/kg daily, *sc*) increased epithelial and endothelial cell apoptosis in tumors without affecting hyperproliferation, indicating that zoledronate is not anti-mitotic at that concentration.[176] Further analyses in the same study suggested a cellular and molecular mechanism involving inhibition of MMP-9 expression and activation of tumor infiltrating macrophages, thereby reducing association of VEGF with its receptor on endothelial cells.[176] In a clinical study, cancer patients with advanced solid cancer and bone metastases were treated with a standard single administration of 4 mg zoledronate. In treated patients, a statistically significant decrease in the serum levels of the circulating angiogenic factors VEGF and PDGF was detected. Zoledronate clearly induced a long lasting effect because even 21 days after the first infusion of zoledronate VEGF serum levels remained significantly below basal values.[181] These studies suggest a variety of mechanisms that may elucidate the anti-angiogenic activities of zoledronate, however the precise molecular and cellular mechanisms responsible for these effects in the *in vivo* studies are still unclear.

2.2.4.2.2 Immunomodulatory effect

In addition to anti-angiogenic effects, zoledronate is thought to modulate the immune system to target and eliminate cancer cells. The immune modulatory activities of zoledronate include stimulation of proliferation and activation of the Vγ9Vδ2 subset of

γδT cells. T cells expressing the Vγ9Vδ2 T cell receptor play a significant role in immune system surveillance and defense.[182] In fact, these cells have potent ability to recognize and kill tumor cells in an MHC-independent manner, suggesting their potential utility in elimination of cancer cells with poor antigen presentation capacity.[183] γδT cells are known to be stimulated by IPP which is an intermediate metabolite of FPP synthesis in the mevalonate pathway. (see Figure 2.9) Internalization of zoledronate by cancer cells leads to inhibition of FPP synthase, resulting in intracellular accumulation of IPP.[184] *In vitro* studies with breast cancer and myeloma cells showed that zoledronate treated cells accumulated phosphorylated mevalonate metabolites (IPP) and then presented those IPP ligands to TCR-γδ cells by an as yet unidentified mechanism.[185,186] Presentation of IPP to γδT cells was shown to stimulate proliferation and subsequent activation of those cells. Consequently, cell lines pre-treated with 5 μM zoledronate showed a marked increase in sensitivity to lysis by activated γδT cells.[187] Several pre-clinical studies have demonstrated that Vγ9Vδ2 T cells expanded *in vitro* sustain their anti-cancer activity *in vivo* upon adoptive transfer into nude mice transplanted with various human cancer cells along with zoledronate treatment.[188-190] Furthermore, clinical studies with cancer patients also support the results of preclinical studies by demonstrating *in vivo* expansion and activation of Vγ9Vδ2 T cells to a subset of IFN-γ producing effector T cells in patients treated with zoledronate, either alone or in combination with low-dose IL-2.[191] Besides cancer cells, monocytes treated with zoledronate were also shown to accumulate IPPs and stimulate proliferation and cytotoxic activation of human Vγ9Vδ2 T cells. Notably, activation of γδT cells requires cell-to-cell contact with zoledronate treated tumor cells or monocytes.[186]

Activated γδ T cells might exert an anti-cancer activity by various mechanisms. As a response to zoledronate mediated activation, γδ T cells secrete TNF-α and IFN-γ which stimulate anti-tumor activity of APCs (NK cells, dendritic cells , macrophages) and αβ T cells.[192] Another mechanism through which zoledronate exerts γδT cell mediated cytotoxicity includes secretion of perforin and granzymes, causing direct tumor cell cytotoxicity.[193] Vγ9Vδ2 lymphocytes also express the activating NK cell receptor

NKG2D which is important for cancer cell recognition by zoledronate activated γδT cells. Through this receptor γδT cells recognize the cancer cells that express the stress inducible MICA/MICB proteins.[194]

Apparently, zoledronate could have a noticeable effect on the γδT cell mediated immune response which might contribute to their *in vivo* anti-tumor activity. However, those findings do not help to explain the mechanism of anti-tumor activity of zoledronate in mouse tumor models because the Vγ9Vδ2 T cell subset exists only in primates. Currently it is unclear if an analogous T cell subset exists in rodents.

Although, all those indirect anti-tumor effects were demonstrated *in vitro*, it is difficult to assess *in vivo* if they are associated with osteoclast inhibitory effects of zoledronate.

2.2.4.2.3 Inhibition of the release of bone derived growth factors

Considering the high concentrations of zoledronate reached in the bone-microenvironment and its inhibitory effect on osteoclast activity, it is conceivable that anti-tumor effects of zoledronate are likely to be mediated indirectly through the inhibition of bone resorption. Reduced bone resorption results in decreased release of bone-derived growth factors that are necessary for the survival of metastatic tumor cells, thereby creating a less favorable microenvironment for the invasion and survival of metastatic tumor cells.

Devastating bone destructions are common in patients with various advanced cancers like breast, prostate, kidney and lung cancer. Those tumors often have destructive localized or systemic effects on the skeleton. Localized effects are mediated by bone-residing metastatic deposits and lead to bone destruction, bone formation or both. On the other side, systemic skeletal destructions do not require presence of bone-residing tumors. These effects are mediated via primary tumor released circulating cytokines and other factors.[195] To stimulate systemic and/or local bone destruction, tumor cells secrete multiple factors such as parathyroid hormone-related peptide (PTHrP), IL-11, IL-6, TNF, RANKL and TGF-α which potently induce osteoclastic activity. Early studies revealed that the majority of patients with solid tumors have increased plasma PTHrP

concentrations. In parallel, women with PTHrP-positive primary breast tumors are more likely to develop bone metastases.[196] PTHrP and other tumor-released factors induce osteoclastic bone degradation that leads to subsequent release of bone-bound growth factors. Bone is a rich reservoir of growth regulatory substances (e.g. transforming growth factors (TGF-β), insulin-like growth factors (IGFs), fibroblast growth factors (FGF), bone morphogenic proteins (BMP), platelet-derived growth factors (PDGFs) and others) which are released during osteoclastic bone resorption.[197] Those growth factors, in turn, stimulate tumor cell proliferation and production of more osteolytic factors, which further promote osteoclastic bone resorption. Thus, reciprocal interactions between cancer cells and the microenvironment create a self-perpetuating circle of bone destruction and tumor growth that is called "vicious cycle of bone" (Figure 2.10).[198]

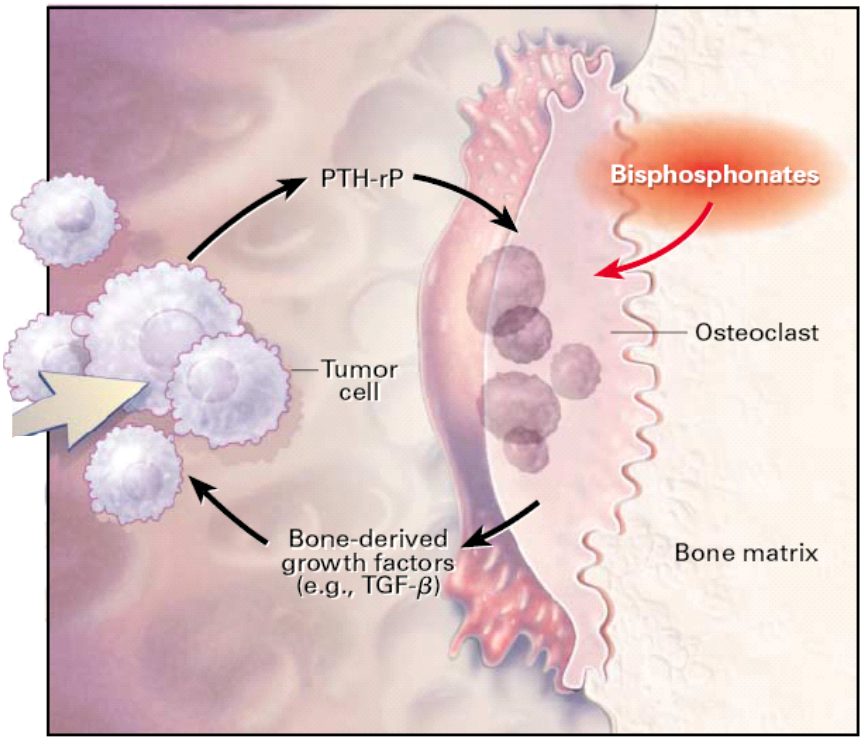


Figure 2.10: Cancer cells secrete PTHrP as the primary stimulator of osteoclastogenesis as well as other factors e.g. IL-6, prostaglandin E2 (PGE2), TNF and M-CSF increasing formation of osteoclasts. During bone resorption, osteoclasts release TGF-β, insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), and bone morphogenetic proteins (BMPs), which further induce the production of PTHrP by tumor cells, as well as growth factors that support tumor growth. This represents the vicious circle that promotes bone destruction and tumor growth. Bisphosphonates break this vicious cycle by inhibiting osteoclast activity in bone. Adapted from Mundy and Yoneda, 1998.[199]

By inhibiting osteoclastic activity, zoledronate was shown to reduce the local release of growth factors from bone and break this self-perpetuating stimulatory mechanism, resulting in a less hospitable environment for tumor growth.[200] Among these growth factors, TGF-β is known as the most abundant cytokine in bone and considered as the main bone-derived factor responsible for driving this vicious cycle of bone metastasis. All three isoforms of TGF-β are present in bone matrix in their latent form. During osteoclastic resorption, the pH is decreased and an acidic microenvironment activates TGF-β in bone.[201] Activated TGF-β is released from mineralized bone matrix and in turn induces production of tumor-derived osteolytic factors.[202,203] Subsequently, tumor derived bone resorption might result in higher levels of TGF-β in the peripheral blood. In cancer patients, higher serum concentrations of TGF-β were detected at later stages of cancer. Elevated serum levels of TGF-β were shown to correlate with enhanced bone metastasis and poor clinical outcome in the late stage of colorectal carcinoma, lung cancer and melanoma.[204-206] Yin J. et al. showed that blockade of TGF-β signaling in breast cancer cells resulted in decreased osteolysis, less tumor burden in bone and enhanced survival in mice bearing tumors.[207] In a study with breast cancer bearing mice, Kang et al. demonstrated that mice treated with BPs (zoledronate or pamidronate) showed less destruction of bone matrix and accordingly a reduced activation of the TGF-β signaling pathway in cancer cells of bone metastases. The authors proposed that these effects might be associated with lowered bioavailability of active TGF-β in bisphosphonate treated mice. The study further revealed that although it does not affect TGF-β signaling in cancer cells *in vitro*, zoledronate can hinder TGF-β signaling *in vivo* by inhibiting osteoclast activity and consequently preventing release of TGF-β from bone matrix.[200] These data suggest that modulation of bone derived factors like TGF-β might also be a possible mechanism responsible for the anti-tumor activity of zoledronate. However, whether zoledronate mediated inhibition of bone derived factors could also exert such an indirect anti-tumor effect in peripheral tissues remains unclear.

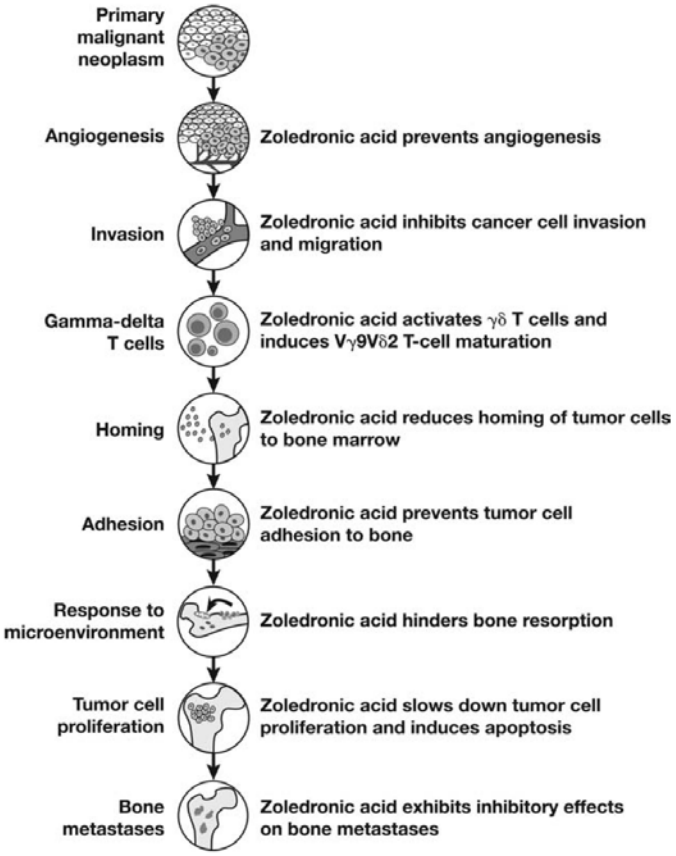


Figure 2.11: *In vitro* and *in vivo* anti-tumor effects of zoledronate. Zoledronic acid was shown to inhibit angiogenesis, cell invasion, cell adhesion, bone metastasis, bone resorption, and cell proliferation as well as activating Vγ9Vδ2 T cells. Lipton et al, 2011.[208]

All together, pre-clinical studies suggest diverse anti-cancer effects of zoledronate in different tumor models (Figure 2.11). Furthermore, clinical studies showed that zoledronate prolongs disease-free survival in cancer patients.[209] However, an exact mechanism by which zoledronate prevents disease progression is still a topic of investigation. Certain other issues still remain uncovered. Does the clinically relevant dose of zoledronate prevent tumor progression in soft tissue tumors and if so, what are the mechanisms underlying this anti-tumor function? Identification of new cellular targets and further elucidation of the cellular and molecular mechanisms by which zoledronate mediates anti-tumor effects would be useful in the design of new therapeutic strategies to modulate and potentiate the anti-tumor effects of this compound.

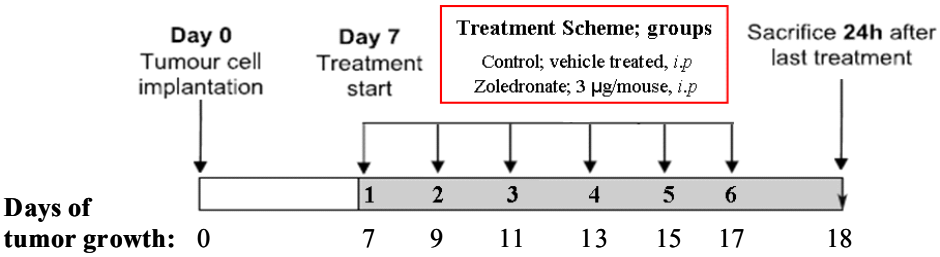
3. RESULTS

3.1 Zoledronate induces apoptosis and delays tumor growth in syngeneic mouse tumor models

Due to its high affinity for bone matrix, zoledronate has been extensively studied for its activity against tumors located in bone and against bone metastases. Recently, a few studies reported growth inhibitory effect of zoledronate on soft tissue tumors. Although the target cells and exact mechanism of action are still unknown, recent studies suggest a possible involvement of myeloid cells in the antitumor activities of the drug. On the basis of these recent findings, we aimed to evaluate the potential immuno-modulatory and anti-tumorigenic properties of zoledronate in syngeneic mouse tumor models. As an initial step to this end, we first evaluated the anti-tumor effects of zoledronate in immunocompetent C57BL/6 mice that were inoculated subcutaneously with Lewis Lung Carcinoma (LLC) cells, and then treated repeatedly with either zoledronate or vehicle control during the course of tumor growth.

To evaluate the therapeutic potential as well as the antitumor activity of zoledronate, the treatment was started only when tumors had reached a size of about 50 mm^3, which was approximately 7 days after tumor cell implantation (Figure 3.1A). Further to this end, zoledronate was applied at clinically relevant doses (i.e., 3 μg/mouse).[210] On average, each mouse received six injections of the drug (a cumulative dose that did not exceed 18 μg throughout the tumor growth analysis) and the animals were monitored for tumor growth and body weight. Mice tolerated the dose well and showed no apparent significant adverse effects. After an initial lag phase, LLC tumors showed rapid growth. However, in comparison to vehicle treated mice, those treated with zoledronate exhibited a significant delay in tumor growth as shown in Figure 3.1B. At the end of the treatment period, the average size of LLC tumors in zoledronate-treated mice was 60% smaller than those in the control group. The tumor growth reduction effect of zoledronate was an early event, already apparent after the first three courses of drug application.

A.



B.

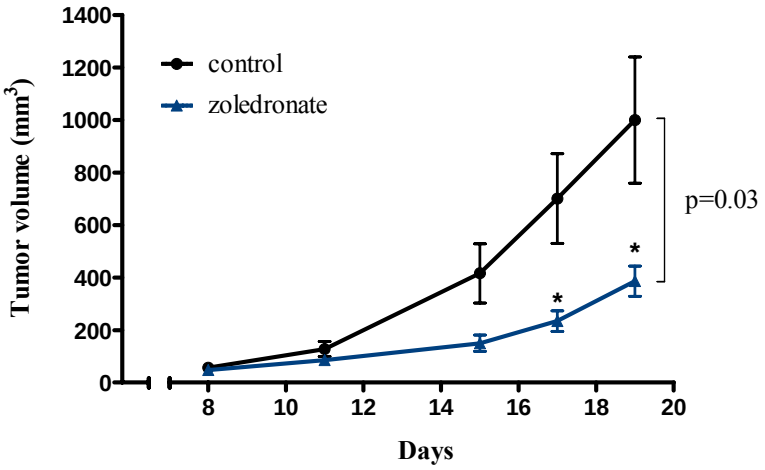


Figure 3.1: Zoledronate administration impairs subcutaneous LLC tumor growth. (A) Scheme of tumor inoculation and zoledronate treatment protocol. C57/BL6 female mice were subcutaneously inoculated with LLC cells ($5x10^5$) suspended in 50 µl HBSS at day 0. When LLC tumors reached a volume of ~50 mm^3 (around day 7), mice were randomized to receive *i.p* injections of zoledronate (3µg in 100µl HBSS) or vehicle (100 µl HBSS) as a control. Treatments were repeated every other day until sacrifice. **(B)** Tumor size was measured once every second or third day and tumor volume was calculated as described in Material and Methods. Tumor growth expressed as mean tumor volumes ±SEM for each treatment group was significantly delayed in zoledronate treated animals. *P<0.05. Data shown is representative of five independent experiments with similar results.

To further evaluate whether other tumor types could respond to zoledronate in a similar way, two different syngeneic tumor models, namely melanoma (B16) and colon adenocarcinoma (MC38), were also tested. As shown in Figure 3.2A, significant inhibition of MC38 tumor growth was observed in treated animals, though the effect was less pronounced than for LLC tumors. However, treatment of B16 melanoma-bearing mice with zoledronate resulted in a moderate but non-significant reduction of tumor growth with respect to the control group. (Figure 3.2B)

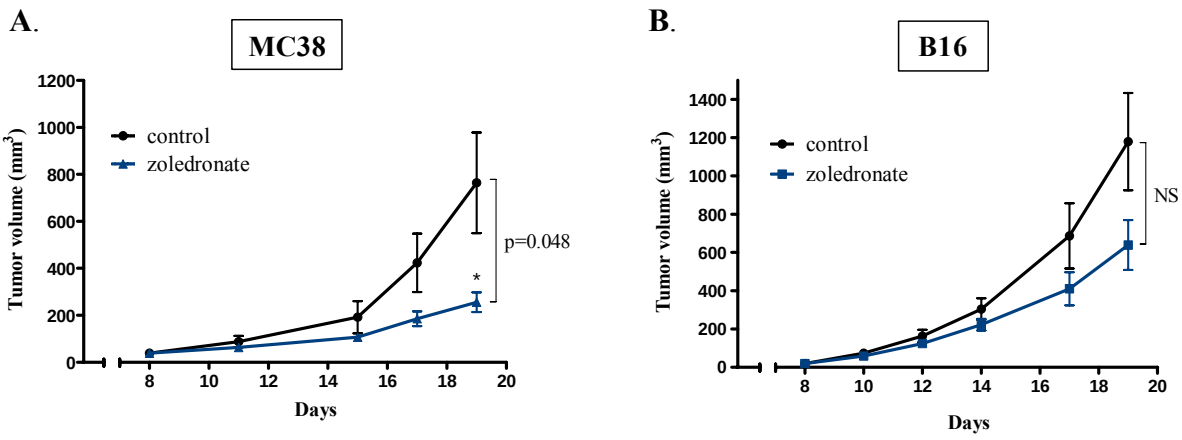


Figure 3.2: Zoledronate significantly restricts tumor growth in the syngeneic mouse tumor models MC38 colon carcinoma but not in B16 melanoma. C57/BL6 female mice were subcutaneously inoculated with MC38 **(A)** or B16 **(B)** cells ($3x10^5$) suspended in 50 µl HBSS at day 0. When tumors reached a volume of ~50 mm^3, mice (n=7 per group) were treated with zoledronate (3 µg/mouse, *i.p.*) or HBSS (control) according to the schedule shown in Figure 3.1A. Treatment was started on day 8 and continued every 2nd day until sacrifice. Results are presented as mean tumor growth ±SEM. *P<0.05. Data are representative of three independent experiments.

To investigate the mechanism underlying the growth inhibitory activity of zoledronate, the potential anti-proliferative and pro-apoptotic properties of the drug were examined in the LLC tumor model. Sections of tumor samples were analyzed for apoptotic and mitotic incidences. To this end, immunostaining for the mitosis-specific antibody anti-phosphohistone H3 was performed in order to detect proliferating cells. As a marker for apoptosis, the activated form of caspase-3, an effector of the apoptotic program, was monitored by IHC. Microscopic analysis revealed that zoledronate treatment had no impact on tumor cell proliferation *in vivo* (Figure 3.3A). However, a marked increase was observed in the number of apoptotic cells in zoledronate treated tumors as compared with vehicle treated tumors (Figure 3.3B). As the systemic concentrations of zoledronate that are achievable *in vivo* do not have a cytotoxic effect on cancer cells *in vitro* [211,212], stromal rather than cancer cell-autonomous mechanisms might account for increased apoptosis *in vivo*. We therefore sought to clarify whether the

zoledronate-induced tumor apoptosis and growth reduction were caused by microenvironmental stress such as the limitation of nutrients/growth factors/oxygen due to impaired angiogenesis or by cytotoxic immune response.

Tumor-associated angiogenesis which is mediated, in part, by tumor infiltrating myeloid cells plays a crucial role in tumor growth and metastasis. Because bisphosphonates have been reported to have anti-angiogenic properties and thereby interfere with tumor growth in several tumor models, we investigated whether the observed growth reduction by zoledronate was associated with inhibition of angiogenesis. IHC analysis of LLC and MC38 tumor tissues was performed in each treatment group to investigate the potential effect of zoledronate on the angiogenic status of the tumors. Anti-CD31 antibody was applied to detect tumor blood vessels. No significant differences were found in the mean vascular density (MVD) of zoledronate-treated LLC or MC38 tumors versus their untreated controls (Figure 3.3C and suppl. Figure 1). This finding suggests that zoledronate impairs tumor growth through mechanisms that are independent of angiogenesis regulation.

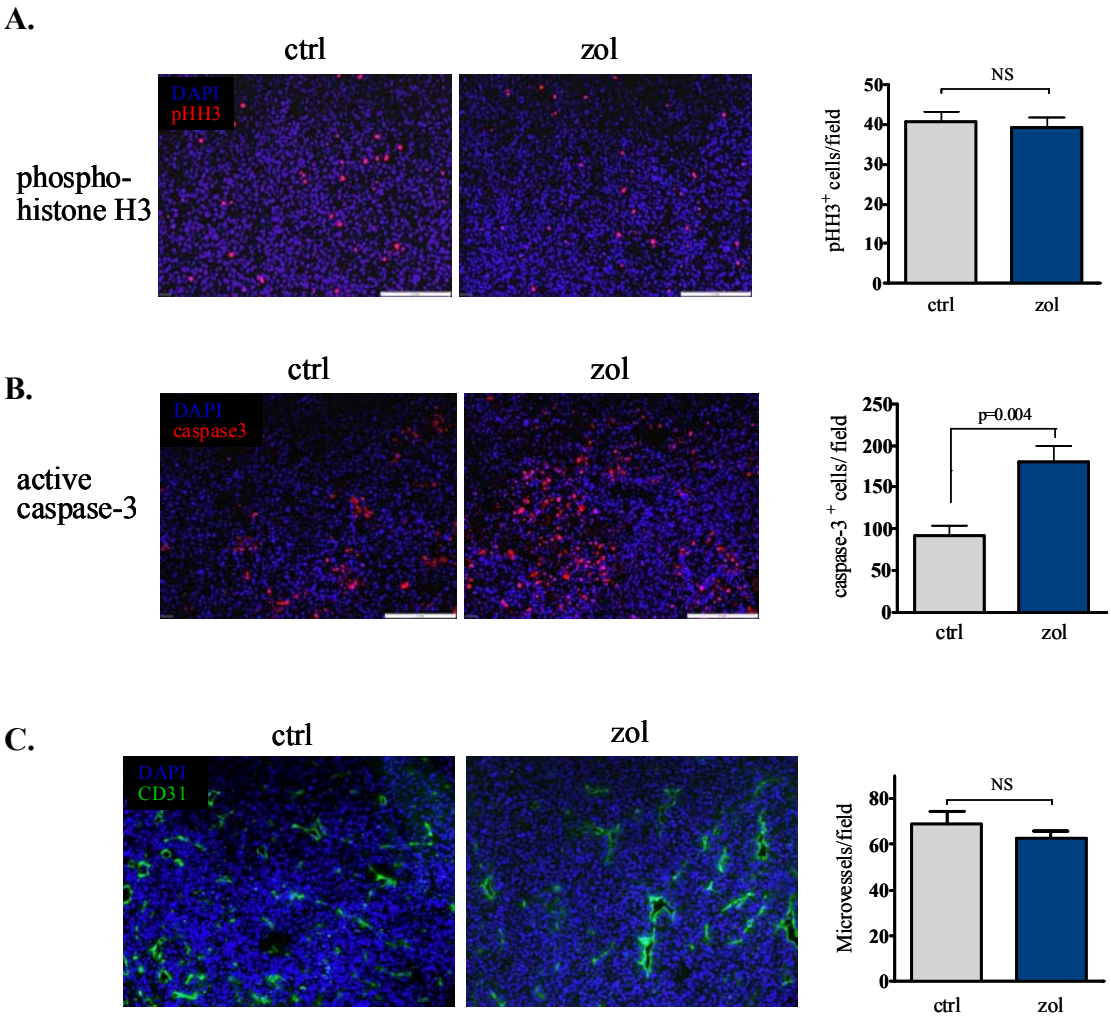


Figure 3.3. Effect of zoledronate treatment on proliferative, apoptotic and angiogenic status of tumors. LLC tumors excised from zoledronate treated and untreated animals were embedded in paraffin, sectioned, and subjected to IHC. Proliferative and apoptotic response of tumors to zoledronate treatment were examined. **(A)** Representative images of pHH3 IHC (left panel). Quantification of pHH3 staining, revealing no changes in the number of proliferating tumor cells in treated and untreated tumors (right panel). **(B)** Representative images of caspase-3 IHC (left panel).Quantification of staining for cleaved caspase-3 revealed increased numbers of apoptotic cells in treated tumors compared to the controls (right panel). Scale bar, 200 μm. Right panels depict mean numbers of $pHH3^+$ or caspase-3^+ cells per field ±SEM (×100 magnification). The number of apoptotic or proliferative cells in a given field (0.8x0.6 mm) was counted for quantitative analysis. In this context, 20 fields per section were counted. Tumors from five different animals were examined for each experimental group of mice. **(C)** Representative images of CD31 IHC (left panel). Right panels depict mean numbers of vessels per field ±SEM (×100 magnification). Microvessels were counted in five randomly selected fields of tumors from four mice of each group. The differences between controls and treated groups were analyzed by unpaired two-tailed Student's *t* test.

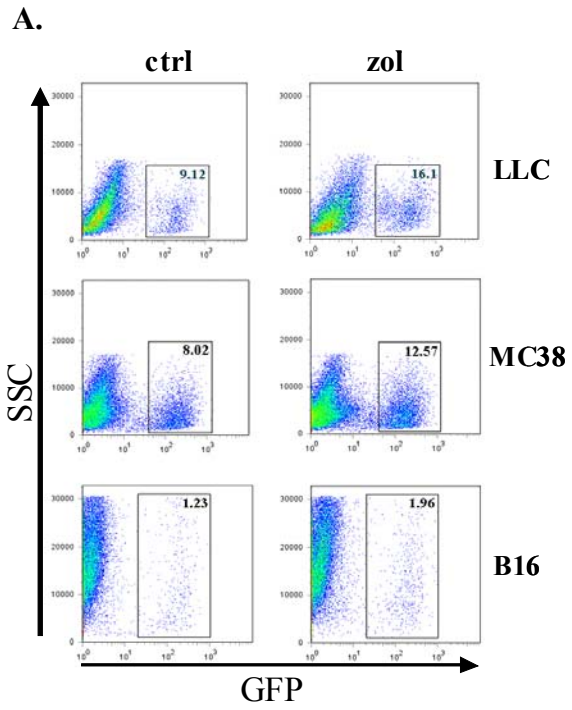
3.2 Zoledronate induces enhanced infiltration of myeloid cells into tumors

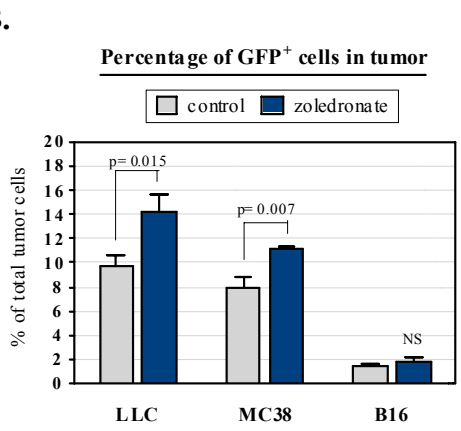
As discussed in section 2.1.2, tumor growth is largely supported by tumor-infiltrating myeloid cells, in particular macrophages and neutrophils, and therefore depletion of these cells is often associated with tumor regression. Due to their high endocytic activity, myeloid cells are in a privileged position to internalize zoledronate. Based on these facts, we predicted an influence of zoledronate on myeloid cells and next set out to investigate whether zoledronate could mediate growth inhibitory effects through the depletion of myeloid cells. Therefore, the frequency of myeloid cells in tumors of treated and untreated animals was analyzed. A GFP reporter mouse model named MacGreen that expresses GFP under the control of the CSF1-receptor promoter in cells of the myeloid lineage was utilized for the thorough detection and isolation of myeloid cells.[213] It is worth noting that as these MacGreen mice express the reporter molecule GFP in cells of the monocyte/macrophage lineage[213] and granulocytes[214], most GFP expressing cells also express CD11b antigen.

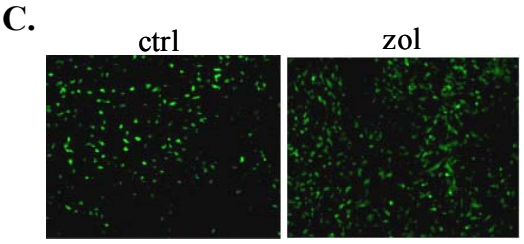
At the end of the treatment period, single cell suspensions prepared from tumors of each individual treated and untreated animals were analyzed by flow cytometry to assess the relative abundance of GFP^+ myeloid cells in the tumor mass. Unexpectedly, no decrease was observed in the frequency of myeloid cells in treated tumors, suggesting that zoledronate does not show cytotoxicity against myeloid cells at the concentrations used in this study. In contrast, the treated tumors surprisingly exhibited higher percentages of GFP^+ myeloid cells than the untreated tumors (Figure 3.4A). Consistent with the flow cytometric data, IHC analyses of tumor sections also confirmed the higher incidence of myeloid cells in zoledronate-treated LLC tumors (Figure 3.4C). A similar pattern was also observed in the MC38 tumor model, but the effect was less pronounced. However, B16 tumors did not exhibit significant changes in the frequency of myeloid cells upon treatment (Figure 3.4A-B). Notably, there was a marked correlation between myeloid cell infiltration in these tumors and the outcome of zoledronate treatment. B16 tumors, which do not exhibit significant growth reduction in response to the drug showed only modest infiltration of myeloid cells (approximately 1.5 % of total cells) when

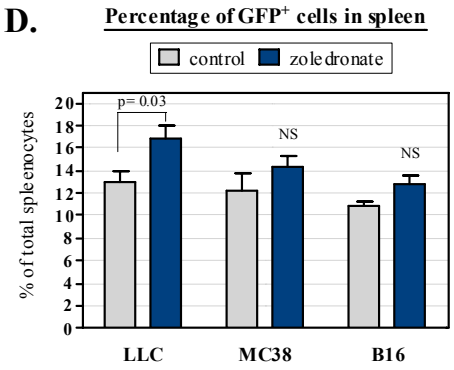
compared with LLC tumors (approximately 10 % of total cells). In the same way, the modest increase in the percentages of myeloid cells in MC38 tumors correlates with the comparatively limited growth inhibitory effect of the drug in this model (Figure 3.4 A-B). The finding of an increased frequency of myeloid cells in the treated tumors prompted us to analyze in turn the frequency of myeloid cells in the bone marrow, blood and spleen harvested from control and treated tumor-bearing mice. After intake, zoledronate is deposited mainly in the bones and as a consequence of osteoclast resorption, is released into the bone marrow wherein it might stimulate the proliferation of myeloid progenitors or their mobilization to peripheral blood. To examine this possibility, bone marrow cells of zoledronate-treated LLC tumor-bearing mice were analyzed for the enrichment of myeloid progenitors. However, evaluation of myeloid cells of treated versus untreated mice revealed that zoledronate treatment did not significantly affect the frequency of myeloid cells in the bone marrow (41.6 ± 4.59 % in control versus 44.13 ± 1.61 % in zoledronate treated mice, Figure 3.4G). A small but significant difference was found in the blood, with the frequency of GFP^+ myeloid cells being slightly higher in treated compared to untreated mice (Figure 3.4F). In the spleen and peritoneum however, percentages of myeloid cells were notably increased in zoledronate-treated mice (Figure 3.4D-E). These results suggest an altered recruitment of these cells or a change in their endogenous migratory capabilities of these cells in zoledronate treated animals.

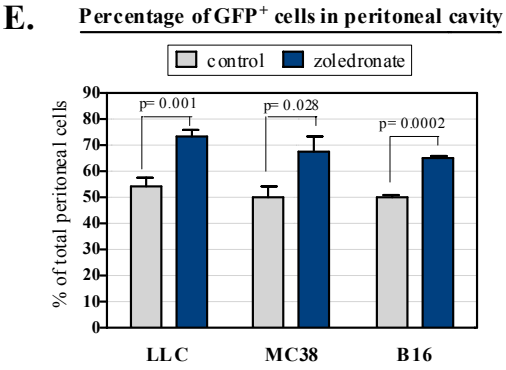
The counterintuitive correlation of enhanced myeloid cell infiltration with the delayed tumor growth observed in zoledronate-treated mice suggests a causative relationship between myeloid cells and the anti-tumor effect of the drug. Therefore, to investigate the possible role of myeloid cells in zoledronate-induced growth delay, these cells were examined in relation to activation status.

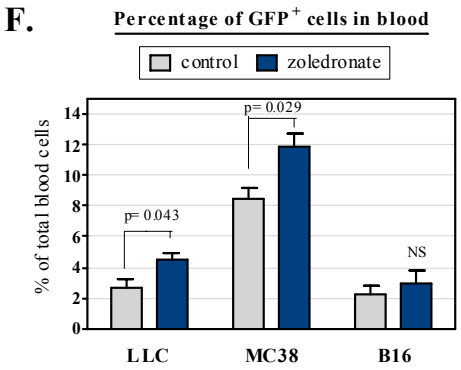

A.
ctrl
zol
LLC
MC38
B16
SSC
GFP
9.12
16.1
8.02
12.57
1.23
1.96

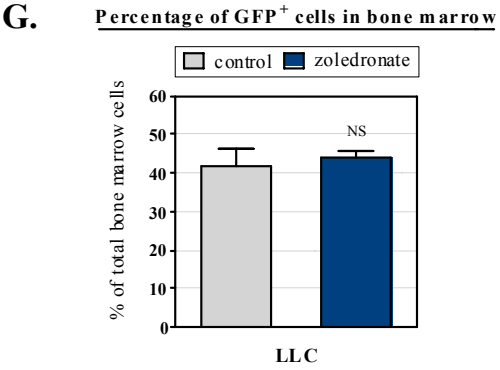

B.
Percentage of GFP+ cells in tumor
control
zoledronate
% of total tumor cells
p= 0.015
p= 0.007
NS
LLC
MC38
B16


C.
ctrl
zol


D.
Percentage of GFP+ cells in spleen
control
zoledronate
% of total spleenocytes
p= 0.03
NS
NS
LLC
MC38
B16


E.
Percentage of GFP+ cells in peritoneal cavity
control
zoledronate
% of total peritoneal cells
p= 0.001
p= 0.028
p= 0.0002
LLC
MC38
B16


F.
Percentage of GFP+ cells in blood
control
zoledronate
% of total blood cells
p= 0.043
p= 0.029
NS
LLC
MC38
B16


G.
Percentage of GFP+ cells in bone marrow
control
zoledronate
% of total bone marrow cells
NS
LLC

3.3 Myeloid cells from zoledronate-treated mice are skewed towards an antitumor phenotype

Although intra-tumoral accumulation of myeloid cells has been widely reported to be associated with enhanced tumor growth,[100,215] in our experiments increased accumulation of myeloid cells in zoledronate-treated tumors was found to be correlated with delayed tumor growth. This suggests that zoledronate may induce changes in the activation status of myeloid cells which transforms them into anti-tumorigenic types. As discussed in the previous section, anti-tumorigenic activation of myeloid cells is associated with changes in the expression patterns of various inflammatory mediators, and also manifested through differential expression of certain (e.g. M1 or M2 related) cell surface antigens. To assess whether zoledronate induces such an anti-tumorigenic phenotype in myeloid cells, GFP^+ cells isolated from both LLC and MC38 tumors of zoledronate-treated and untreated mice (Figure 3.5A) were subjected to real-time RT-PCR for the analysis of differential expression of relevant M1 and M2 activation state markers, which represent anti/pro-tumorigenic phenotypes respectively. Not only the tumor infiltrating but also the peritoneal myeloid cells were analyzed for their differential activation status. This was because, peritoneal myeloid cells of tumor-bearing mice are known to exhibit a pro-tumorigenic phenotype which is characterized by the increased expression of immunosuppressive cytokines as well as reduced expression of immunogenic factors.[114]

Figure 3.4: Zoledronate causes changes in the extent of myeloid cell infiltration in tumor-bearing mice The s.c. LLC, B16 and MC38 tumor-bearing MacGreen mice were either treated with zoledronate or vehicle (n=6-8/group; i.p.) as described in Materials and Methods. Mice were sacrificed 24h after the last treatment, and tumors were harvested and processed. Percentages of GFP^+ cells in LLC, B16 and MC38 tumor cell suspensions were analyzed by flow cytometry **(A)** shows a representative tracing of GFP^+ cells in tumors of each cell lines. The numbers in each square represent the percentage of GFP^+ cells in the tumor mass. **(B)** summarizes percentages of GFP^+ cells in both treated and untreated groups. **(C)** IHC of LLC tumors, confirming enhanced infiltration of GFP^+ myeloid cells (green) into tumors of treated mice. Sections are representative of multiple samples from more than four experiments. **(D-G)** summarizes percentages of GFP^+ cells in blood, spleen and peritoneal lavage fluid of mice bearing tumors of each cell lines **(D-F)** and in the bone marrow of LLC bearing mice **(G)**. Data are representative of three independent experiments and show mean ± SEM of 6-8 mice. Statistical significance was assessed by two-sided Student's t-test.

As shown in Figure 3.5B, quantitative real-time PCR data revealed a marked decrease in expression of the immunosuppressive cytokines IL-10 and arginase-1 in the GFP^+ myeloid cells of zoledronate-treated tumors. On the other hand, a simultaneous increase in the expression of the immunostimulatory factors IL-12, IFN-γ and iNOS suggests an anti-tumorigenic reprogramming of these cells. Furthermore, the acquisition of a M1-like phenotype by myeloid cells from treated tumors was reflected by their increased expression of MHC class II molecules as well as by a decreased expression of the scavenger receptor CD163, which has recently been proposed as a marker to characterize the pro-tumorigenic activated M2-type macrophages.[216,217] IHC analysis of tumor tissue sections further demonstrated that even though the total number of myeloid cells was increased, the number of CD163 positive myeloid cells was significantly decreased in treated tumors (suppl. Fig.3.2). Peritoneal myeloid cells from treated mice also exhibited a comparable polarization from M2 to M1 phenotype (Figure 3.5C). Evidently, zoledronate treatment skewed the myeloid cells from an M2-like immunosuppressive phenotype to an M1-like immunostimulatory phenotype.

To further evaluate the immunostimulatory capacity of myeloid cells from treated tumors, we tested their effect on $CD8^+$ T cell proliferation and activation *in vitro*. GFP^+ myeloid cells isolated from tumors of treated or untreated animals were co-cultured with fluorescent dye CFSE labeled $CD8^+$ T cells isolated from spleens of non-tumor bearing naïve animals. As expected and as shown in Fig. 3.6A-B, myeloid cells from zoledronate-treated tumors showed enhanced capacity to stimulate allogeneic $CD8^+$ T cell proliferation and IFN-γ production.

It is highly probable that these M1-activated immunostimulatory myeloid cells play an important role in the observed effect of the drug on tumor growth.

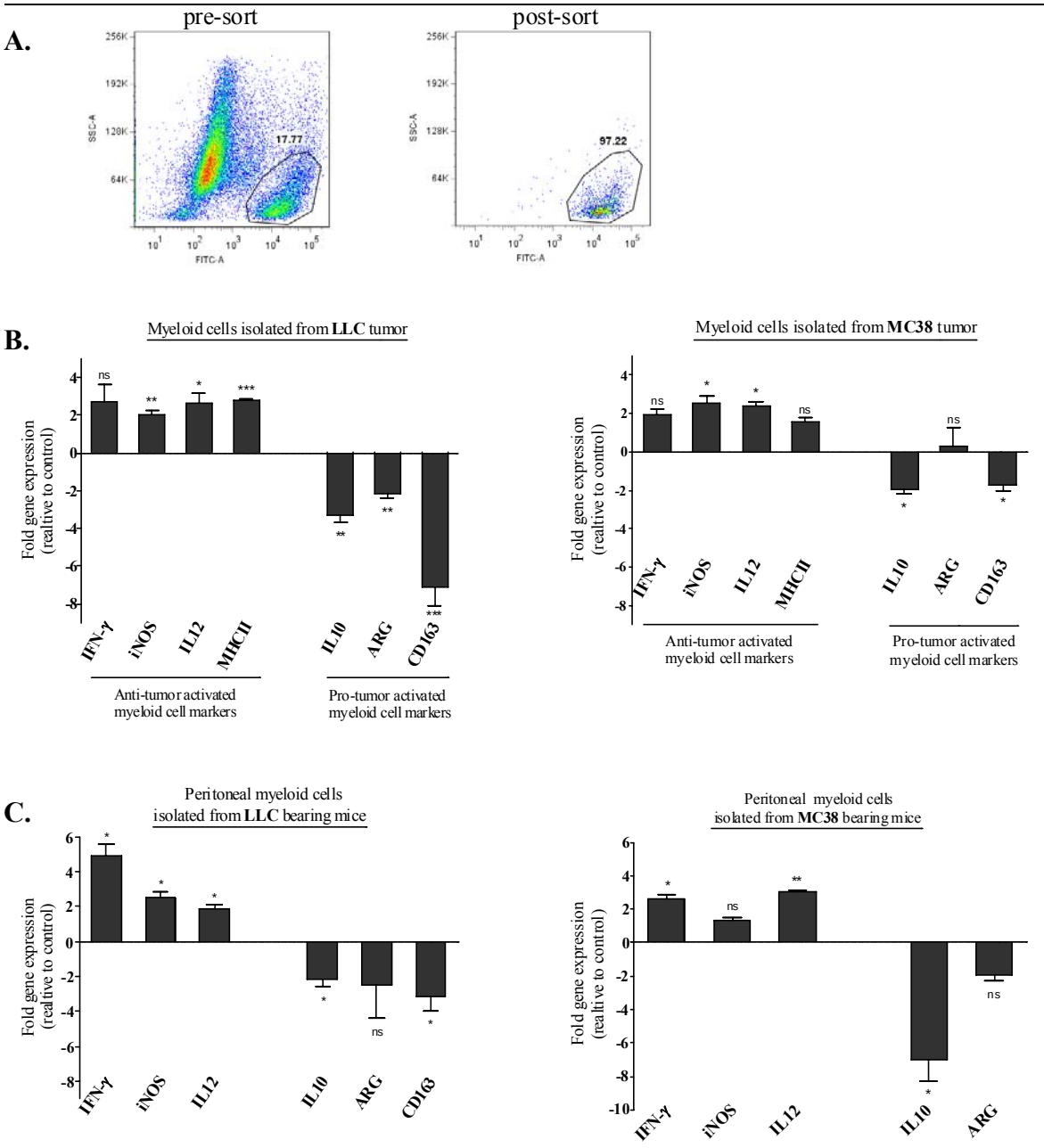


Figure 3.5 Myeloid cells of zoledronate treated mice exhibit a more immunostimulatory gene expression profile. LLC and MC38 tumors were removed from treated or untreated mice at the end of the treatment period. Single cell suspensions were prepared and myeloid cells isolated based on FACS sorting for GFP^+ cells. **(A)** Single cell sorting of GFP^+ cells from tumors of LLC bearing mice. A representative flow cytometric analysis of GFP^+ cells before and after sorting is shown. **(B-C)** RT-PCR analysis revealing that myeloid cells sorted from tumors **(B)** and the peritoneal cavity **(C)** of zoledronate treated LLC and MC38 tumor bearing mice express reduced levels of IL-10, arginase and CD163 and increased levels of IL-12, IFN-γ, MHC-II and iNOS. Results are given as fold increase in mRNA expression relative to that in myeloid cells from untreated mice. Data were normalized to β-actin gene expression and are representative of 3 independent experiments. Graphs represent the mean (±SEM) of four individual tumor samples. The significance was determined by one-sample *t* test (*p<0.05, **p<0.01, ***p<0.001)

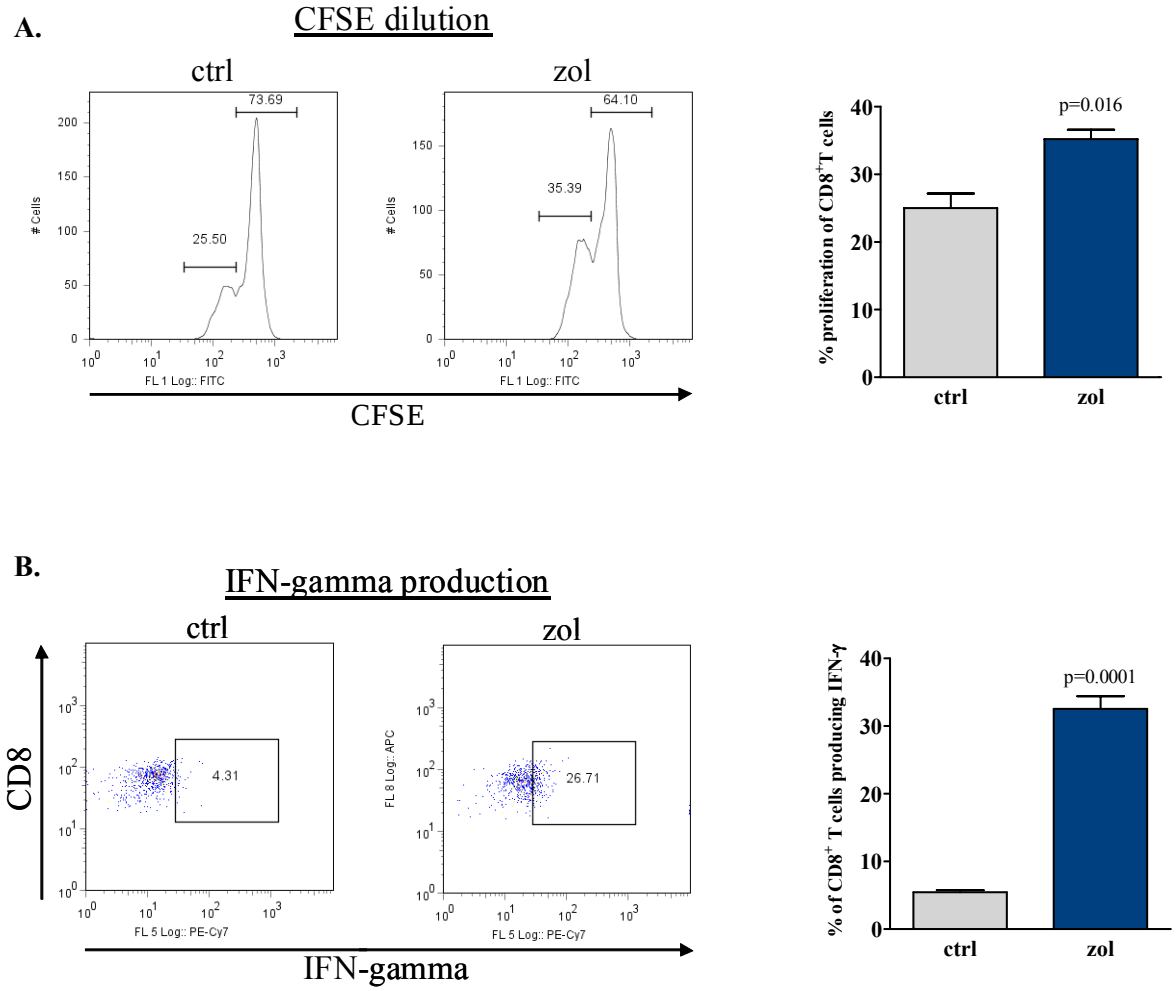


Figure 3.6. Myeloid cells from tumors of zoledronate treated mice increase the proliferation and IFN-γ production of CD8$^+$ T cells *in vitro*. GFP$^+$ cells isolated from tumors of treated and untreated animals were co-cultured with CFSE labeled CD8$^+$ T cells sorted from spleens of naïve mice as described in Material and Methods. A total of 3x10^4 GFP$^+$ cells were cultured with CFSE-labeled CD8$^+$ T cells (1×10^5 cells, ratio 1:3) and incubated at 37°C in 5% CO_2 for 72 h. *In vitro* CFSE proliferation **(A)** and IFN-γ production **(B)** of CD8$^+$ T cells were measured by flow cytometry. Left panels show a representative flow cytometry tracing of CD8$^+$ T cells co-cultured with myeloid cells from treated or untreated tumors. Right panels show mean percentages of proliferating or IFN-γ producing CD8$^+$ T cells out of total CD8$^+$ T cells co-cultured with myeloid cells. Data are representative of 2 independent experiments (n = 3 mice per group). Statistical significance was assessed by two-sided Student's t-test.

3.4 Neutrophils are the augmented subset of myeloid cells in zoledronate treated tumors

Tumors recruit a wide variety of myeloid cell types distinguishable by their functions as well as by their expressions of specific cell surface markers and as discussed earlier, different compositions of myeloid subsets in the tumor microenvironment can give rise to different therapeutic responses by the tumor.[218] With this perception in mind we next set out to characterize the cellular composition of the infiltrating myeloid cells in order to identify the subpopulation(s) differentially accumulating in tumors of zoledronate-treated animals. To this end, single cell suspensions of tumors were analyzed for various myeloid cell surface markers that define macrophages (F4/80), monocytes (CD11b), neutrophils (Ly6G) and dendritic cells (CD11c). As shown in Figure 3.7, no significant changes in the frequency of infiltrating macrophage or dendritic cell populations were observed in response to the treatment. However, a slight increase was detected in the percentages of infiltrating $CD11b^+$ cells. Both neutrophils and monocytes/macrophages express CD11b. To differentiate monocytes/macrophages from neutrophils, the anti-Ly6G antibody (clone 1A8) which is specific for neutrophils was used.[219] Zoledronate treatment led to a significant increase in the percentage of intratumoral $Ly6G^+$ neutrophils in LLC tumors (3.41 ± 0.33 % for zoledronate-treated versus 1.75 ± 0.26 % for controls). B16 and MC38 tumors exhibited only a slight, and non-significant, increase in neutrophil content upon treatment (Figure 3.7).

Evaluation of myeloid cell subsets in spleen and blood of mice treated with zoledronate showed a similar pattern of a slight increase in the percentage of $Ly6G^+CD11b^+$ neutrophils in comparison to untreated mice. However, as predicted, analysis of bone marrow showed no change in the abundance of any of the monocytic or neutrophil cell populations (suppl. Fig. 3). Increased frequency of neutrophils in treated tumors, spleens and blood, but not in bone marrow suggest that zoledronate does not induce the enhanced expansion or differentiation of neutrophils in the bone marrow but that alternatively it induces increased chemoattraction and consequent release of neutrophils from bone marrow, thereby resulting in their increased infiltration into tumors.

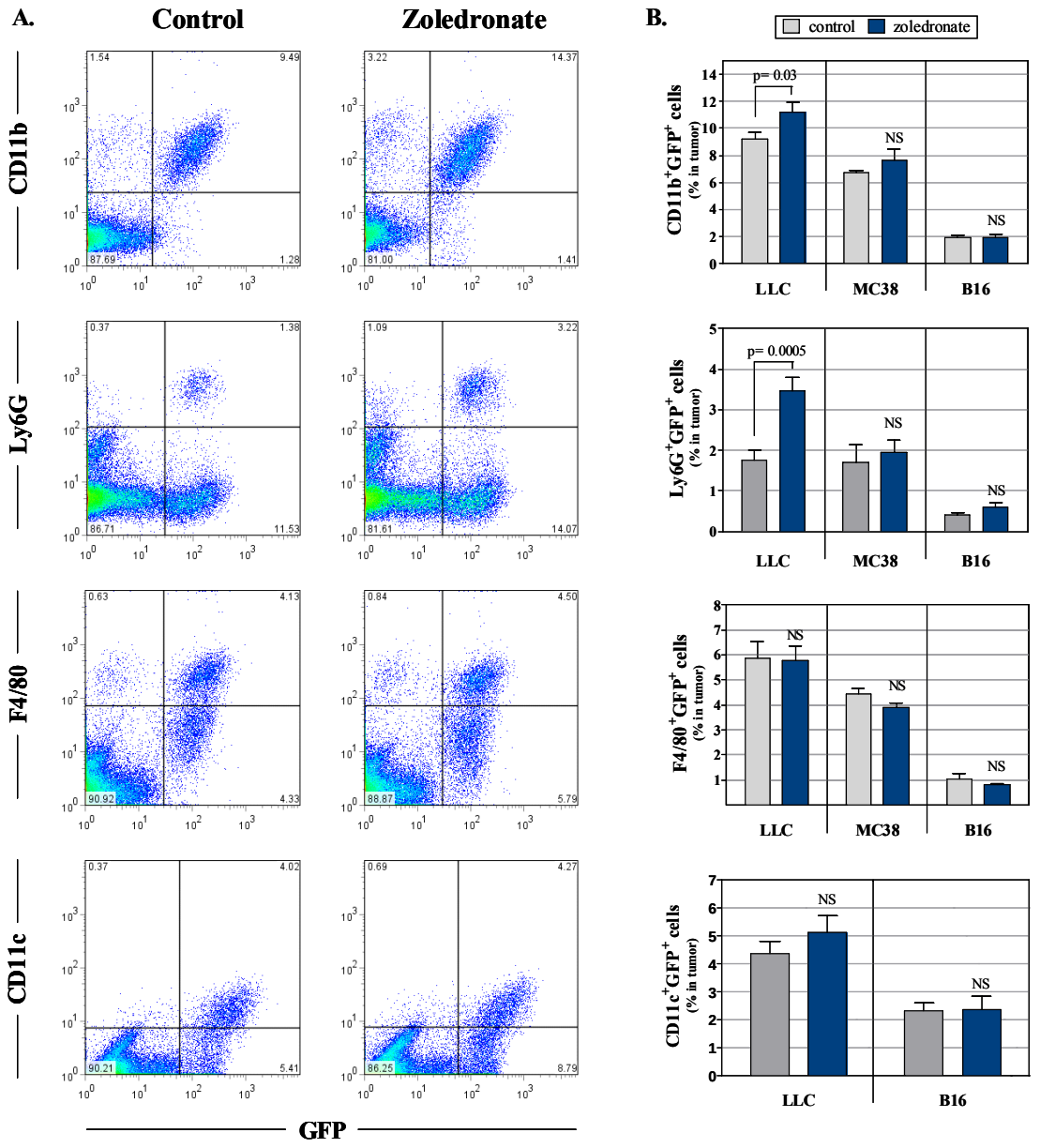


Figure 3.7: Enhanced numbers of Ly6G$^+$ neutrophils in tumors of zoledronate treated mice. Single cell suspensions were prepared from tumors of treated and untreated animals and infiltrating myeloid cell populations were analyzed by flow cytometry. Patterns of tumor-infiltrating myeloid cells in mice, presented as the frequency of infiltrating CD11b$^+$, Ly6G $^+$, F4/80$^+$, CD11c$^+$ cells, gated on GFP$^+$ cells, in zoledronate treated LLC tumors relative to untreated LLC tumors are shown. **(A)** Representative plots of cells derived from LLC tumors of individual mice are shown. **(B)** Relative percentages of monocytes, neutrophils, macrophages, and dendritic cells. Numbers represent the percentage of the relevant population in the tumor mass. Data are representative of 2-3 independent experiments (n = 6-8 mice per group). Statistical significance was assessed by two-sided Student's t-test.

3.5 Zoledronate enhances expression of neutrophil chemoattractants in tumors

Neutrophil recruitment into tumors is tightly regulated by chemokines that are produced by both stromal and tumor cells. The increased neutrophil infiltration into tumors of zoledronate-treated animals suggested a possible increase in the production of neutrophil chemoattractants by these tumors compared to control tumors. This was examined by analyzing the mRNA expression levels of several chemokines (i.e. CXCL1/KC, CXCL2/MIP-2α, CCL3/MIP-1α, and CXCL5/LIX) that play an important role in the recruitment and chemoattraction of neutrophils. As shown in Figure 3.8A, treated tumors exhibited a 2.8- and 4.4-fold increase in the expression levels of CXCL2 and CXCL5, respectively. CXCL5 is frequently described as a bona fide chemokine for neutrophil migration[220] and these results are strengthening our hypothesis that the increase in neutrophil counts in tumors following zoledronate treatment is at least partly due to their increased chemotactic migration. No difference was observed in the expression levels of the two other tested chemoattractants, CXCL1 and CCL3, in zoledronate treated tumors.

Tumors are highly heterogeneous in their cellular composition and these chemokines are known to be produced by distinct cells types in this microenvironment. Therefore we next sought to nail down the cellular sources of these chemokines in treated tumors. Initially tumor-infiltrating myeloid cells were tested as a potential source of neutrophil chemoattractants. Myeloid cells isolated from treated and untreated tumors were analyzed for differential expression of the chemoattractants shown in Figure 3.8B. Myeloid cells from zoledronate-treated tumors exhibited a 2-fold increase in CXCL5 expression but did not show any significant changes in the expression of the other selected chemokines. To investigate whether cancer cells themselves also contribute to elevated expression of neutrophil chemoattractants in treated tumors, we made use of LLC cell line *in vitro*.

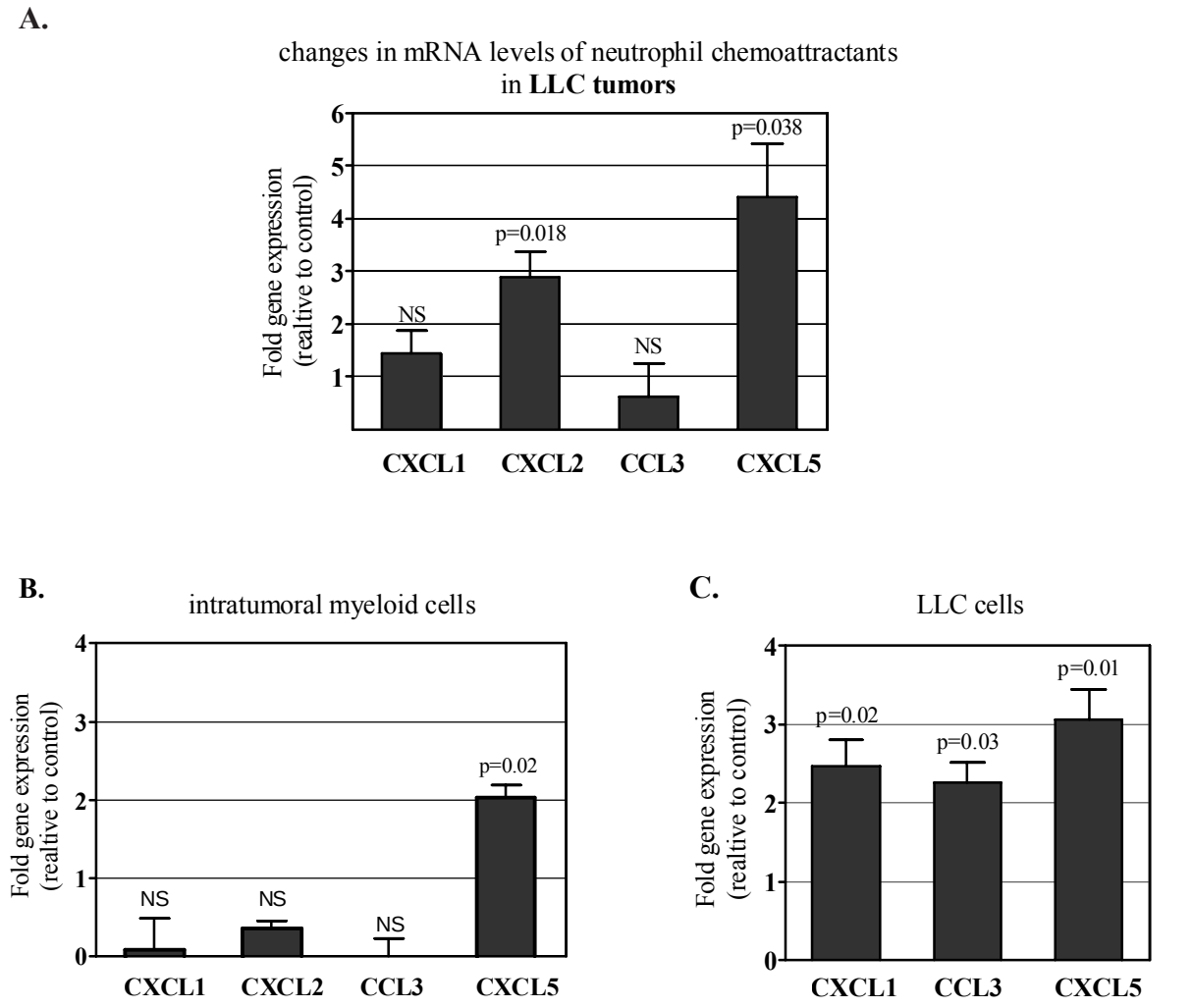


Figure 3.8: Induction of neutrophil attracting chemokines in tumors following zoledronate treatment. (A) LLC tumors (n =5 per group) from treated and untreated mice were harvested and homogenized. RNA was isolated and synthesized cDNA was analyzed using real-time PCR for the expression of relevant neutrophil chemoattractants. Fold changes and p-values are shown, relative to untreated control. **(B)** Myeloid cells were isolated from treated and untreated tumors (n=4 per group) and assayed for the differential expression of neutrophil chemoattractants. Experiments were done twice with at least five animals per group. **(C)** LLC cells ($5x10^5$ cells/well into a 6 well culture plate) were placed in culture either without or with 2 μM zoledronate for 4 hours. cDNA was assayed for chemoattractant expression. The results shown represent three separate experiments. Statistical significance was determined by one-sample t-test.

Expression levels were examined in LLC cells cultured under zoledronate supplemented versus normal culture conditions. As shown in Figure 3.8C, zoledronate led to a significant increase in the expression of CXCL1, CCL3 and CXCL5 mRNA. Expression of CXCL2 mRNA was undetectable probably due to low intrinsic expression.

Although CXCL2 was found to be upregulated in treated tumor tissue, neither LLC nor myeloid cells exhibited increased levels of CXCL2 mRNA. It is possible that other stromal compartments such as epithelial cells or fibroblasts, may be responsible for the observed increase in CXCL2 expression in treated tumor tissue.

These findings suggest that zoledronate could augment the intratumoral recruitment of neutrophils by inducing secretion of chemoattractants, which are at least partially derived from both the myeloid and cancer cell compartments of tumors.

3.6 Zoledronate treatment induces an immunostimulatory profile in intratumoral neutrophils

While tumor associated neutrophils are known to support tumor growth, the inverse correlation observed between neutrophil abundance and tumor growth observed in zoledronate-treated mice suggests that this treatment could induce neutrophils to adopt unique phenotypic and functional properties. Given that as was shown in section 3.3 myeloid cells in general are skewed toward a pro-inflammatory and anti-tumorigenic phenotype by zoledronate, two major subsets of myeloid cells, namely $CD11b^{+}Ly6G^{+}$ neutrophils and $CD11b^{+}Ly6G^{-}$ monocytes/ macrophages were analyzed for changes in their immunostimulatory and immuno-suppressive properties. To this end, intratumoral $CD11b^{+}$ cells were first sorted by FACS to separate the $CD11b^{+}Ly6G^{+}$ neutrophils and $CD11b^{+}Ly6G^{-}$ monocytes/macrophages. The purity of the isolated populations was found to be >90% following isolation (Figure 3.9A). To study the phenotypic changes in the neutrophils and macrophages following zoledronate treatment, real-time RT-PCR analyses of selected enzymes and cytokines were performed on these isolated populations. These analyses revealed that zoledronate treatment affected expression of immunomodulatory genes, particularly in the neutrophil rather than in the macrophage subset of tumor infiltrating myeloid cells. As shown in Fig. 3.9B, with the exception of iNOS, the monocyte/macrophages subset from zoledronate-treated tumors did not exhibit alterations in the message levels of immunostimulatory or immunosuppressive mediators, whereas the neutrophils showed a more pro-inflammatory and immunostimulatory phenotype.

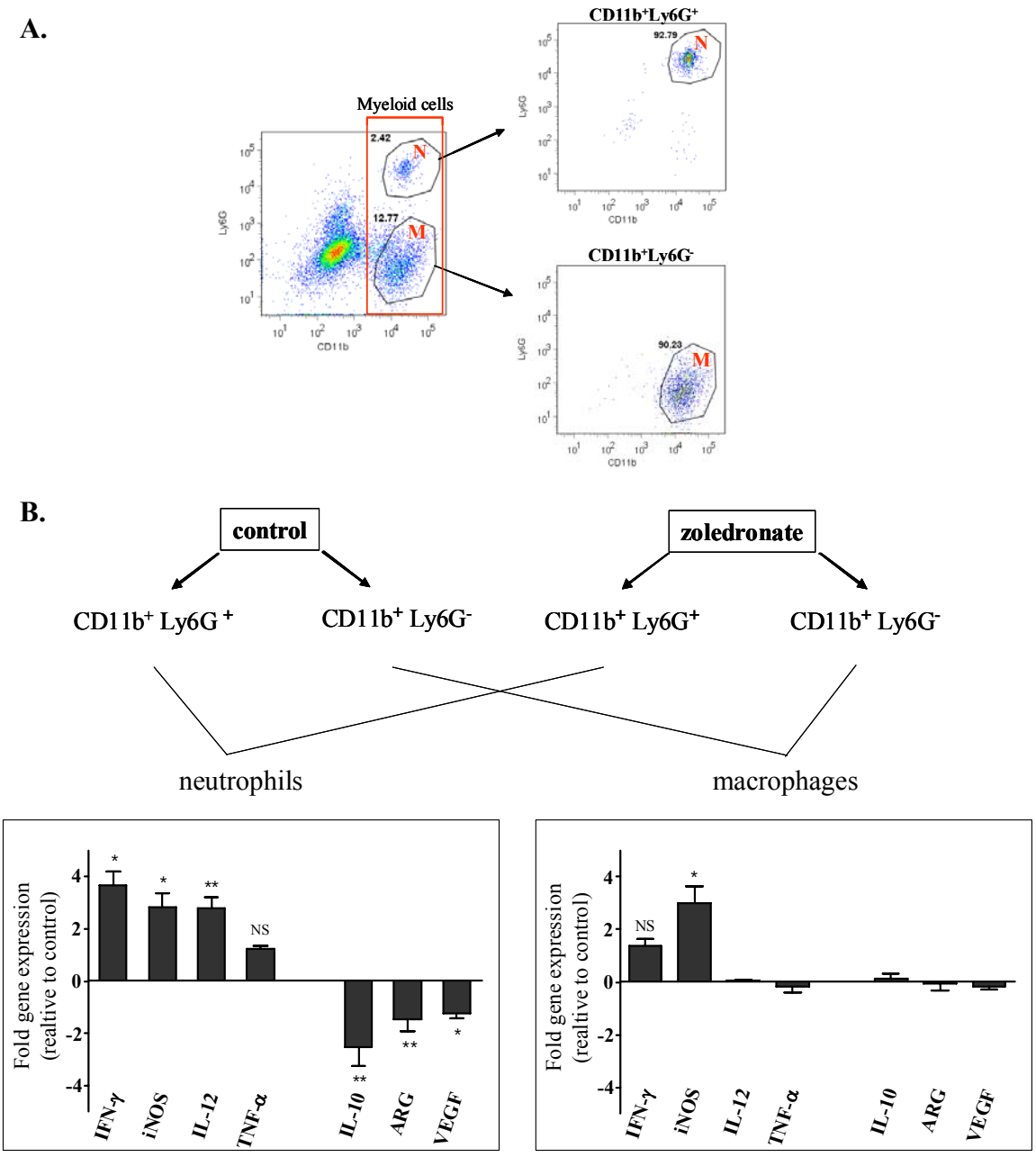


Figure 3.9: Zoledronate promotes an inflammatory and immunogenic gene expression profile specifically in neutrophil subset of myeloid cells (A) Neutrophil ($CD11b^{+}Ly6G^{+}$) and monocyte/macrophage ($CD11b^{+}Ly6G^{-}$) subsets of myeloid cells were isolated from zoledronate treated and untreated LLC tumors (n=6 per group). A representative flow cytometry analysis of $CD11b^{+}Ly6G^{+}$ and $CD11b^{+}Ly6G^{-}$ cells before and after sorting is shown. **(B)** Isolated cell populations were subjected to gene expression analysis by real-time PCR for the representative M1/M2 or N1/N2 chemokines and cytokines. Results were normalized to β-actin levels. Fold change of each factor was calculated using the expression level in the untreated control cell population as the denominator. Data are representative of 3 independent experiments. The significance was determined by one-sample *t*-test (*p<0.05, **p<0.01, ***p<0.001).

In this context, expression levels of the immunosuppressive factors IL-10 and arginase were found to be significantly reduced while levels of the pro-inflammatory and immunostimulatory mediators iNOS, IL-12 and TNF-α were increased in neutrophils from zoledronate-treated tumors. Overall, zoledronate skews neutrophil, but not macrophage, subset polarization away from the N2- to the N1-like phenotype. These findings signify those immunostimulatory, N1 activated neutrophils to be potential mediators of zoledronate-induced antitumor activity.

3.7 Depletion of neutrophils abolishes the growth inhibitory effect of zoledronate

Having ascertained the increased infiltration and anti-tumorigenic activation of neutrophils in treated tumors, the functional importance of these cells in mediating zoledronate-induced growth reduction was tested by depleting $Ly6G^+$ cells in LLC tumor-bearing animals. The LLC tumor model was selected due to its higher abundance of intratumoral neutrophils that corrolates with the stronger growth inhibitory effect of zoledronate in this model compared to MC38 and B16 models (cf. Figures 3.1 and 3.2). Neutrophils were depleted in both treated and untreated tumor-bearing animals by using an i.p. administered neutrophil-specific anti-Ly6G antibody[219] (Figure 3.10A). Tumor growth was compared with that in the control groups which were treated with a nonspecific isotype antibody. Efficiency of depletion was determined by analyzing blood and tumor samples from treated and untreated animals by flow cytometry. Intraperitoneal administration of the anti-Ly6G antibody caused depletion of approximately 90% of neutrophils from blood and tumors (Figure 3.10B). Isotype-matched control antibodies had no effect on tumor growth in control mice. As discussed previously, tumor infiltrating neutrophils in general support tumor growth, and predictably, elimination of neutrophils in untreated control mice resulted in a modest, but non-significant, reduction in tumor growth suggesting that neutrophils from untreated tumors predominantly had a N2-like tumor-promoting phenotype in these conditions. However, depletion of neutrophils in zoledronate-treated mice caused a significant reversal of the growth inhibition induced by the drug. The difference in tumor growth between control and zoledronate-treated mice was no longer observed when mice

were treated with anti Ly6G antibodies (Figure 3.10C). These results corroborate the essential role of neutrophils in zoledronate mediated tumor growth inhibition.

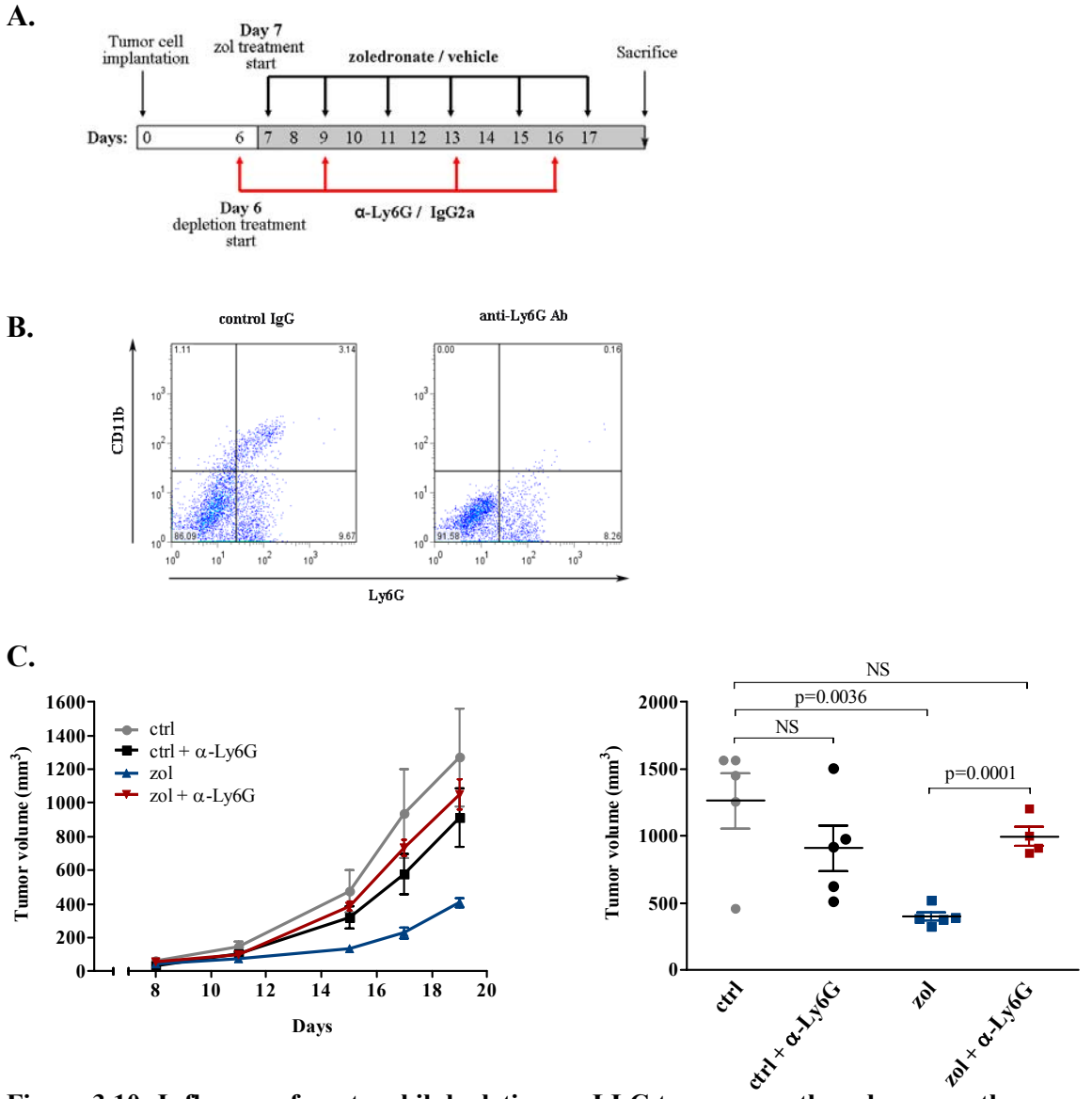


Figure 3.10: Influence of neutrophil depletion on LLC tumor growth and on growth response to zoledronate treatment. Mice bearing LLC tumors were divided into four groups (5-6 mice/group) and treated as follows: no treatment; administration of anti-LyG Ab; zoledronate treatment only; and zoledronate treatment in combination with anti-Ly6G Ab. **(A)** 100 µg of the anti-Ly6G monoclonal antibody (1A8) was first applied one day before the initiation zoledronate treatment and then twice a week over the course of the experiment. The groups not treated with 1A8 received an isotype-matched control antibody at the same schedule and dose. All treatments were applied intraperitoneally. **(B)** At the end of treatment, neutrophil depletion was confirmed by flow cytometric analysis. In **(C)** the differences in mean tumor size ± SEM (left panel) and tumor sizes of individual mice (right panel) with or without Ly6G depletion (α-Ly6G) in zoledronate treated or untreated mice are shown. Data are representative of 2 independent experiments.

3.8 Systemic administration of TGF-β reverses anti-tumor effects of zoledronate

As mentioned before, growing tumors induce resorption of bone that in turn results in release of active TGF-β, thus further stimulating tumor growth. In conjunction, a few studies have shown that bisphosphonates reduce bone derived TGF-β release by inhibiting osteoclast mediated bone resorption. Recent findings highlight the importance of TGF-β signaling in modulation of the immune cells in the tumor microenvironment and in orchestration of tumor development.[221,222] It is known that ablation of carcinoma cell-specific TGF-β signaling leads to increased myeloid cell infiltrates in the tumor microenvironment.[223,224] This recruitment was correlated with increased expression of the chemokines CXCL1 and CXCL5 in the TGF-β signaling deficient tumor tissue.[63,225] Subsequent studies showed that systemic inhibition of TGF-β in tumor-bearing mice led to an influx of neutrophils into tumors. Furthermore, under TGF-β inhibiting conditions *in vivo*, neutrophils were shown to acquire an anti-tumor N1 phenotype.[60] It is notable that the characteristics described above for conditions of TGF-β deficiency bear striking resemblances to those we have identified for zoledronate-treated tumors in the course of our studies.Therefore we next aimed to investigate the relevance of TGF-β in zoledronate driven changes in the tumor microenvironment and tumor growth by first examining the effect of zoledronate treatment on systemic TGF-β levels in LLC tumor-bearing animals. To this end, levels of TGF-β1 were measured in blood plasma samples collected from treated and untreated tumor-bearing mice. As shown in Figure 3.11A, zoledronate treatment induced only a modest and non-significant decrease in the level of systemic TGF-β1. Modulations in bone and plasma TGF-β levels are known to be subtle and/or transitional and therefore, detection would require repetitive measurements using more sensitive detection methods.[226] Nevertheless, these transitional and/or subtle changes in TGF-β levels can affect downstream signaling that is important for neutrophil function, and thereby could be consequential in determining the effect of zoledronate. For these reasons, we went on to employ an indirect approach to assessing TGF-β significance, by determining whether supplementation of recombinant TGF-β (rTGF-β) to zoledronate-treated animals would reverse the effects of the drug. Tumor-bearing animals were left

untreated or treated with zoledronate with or without rTGF-β administration. We first evaluated tumor growth under the influence of TGF-β and assessed the influence of TGF-β on the antitumorigenic effects of zoledronate. Administration of rTGF-β had no effects on animal health as well as tumor growth pattern in control group. However, administration of rTGF-β in zoledronate-treated animals abolished the growth inhibitory effect of zoledronate (Figure 3.11B-C). Since we had shown that zoledronate-induced growth inhibition is mediated by neutrophils we next asked whether neutrophil infiltration was also affected by TGF-β. As shown in Figure 3.11D, rTGF-β administration also reduced the frequency of neutrophils in tumors treated with zoledronate to a level comparable to control tumors. These results not only suggest the possible involvement of TGF-β in zoledronate-induced neutrophil infiltration but also support the hypothesis that neutrophils are important for the effects mediated by zoledronate in these tumor models. To validate further the influence of TGF-β in neutrophil infiltration of tumors in zoledronate-treated animals, *in vitro* assays were conducted. Given that zoledronate induces increased expression of neutrophil chemoattractants in LLC cells (see Figure 3.7), we tested whether TGF-β could suppress the zoledronate-induced neutrophil chemoattractant expression in these cells. In this regard, LLC cells in culture were either left untreated or treated either with zoledronate alone or in combination with rTGF-β. Quantitative RT-PCR analysis showed that while zoledronate treatment increased the expression of neutrophil chemoattractants in LLC cells, rTGF-β supplementation significantly attenuated the effect of zoledronate with regard to up-regulation of CXCL1, CCL3 and CXCL5 expression (Figure 3.11E). Thus, this suppression of chemokine expression by supplementation with TGF-β suggests that impairment in TGF-β signaling would results in an enhancement of chemokine expression.

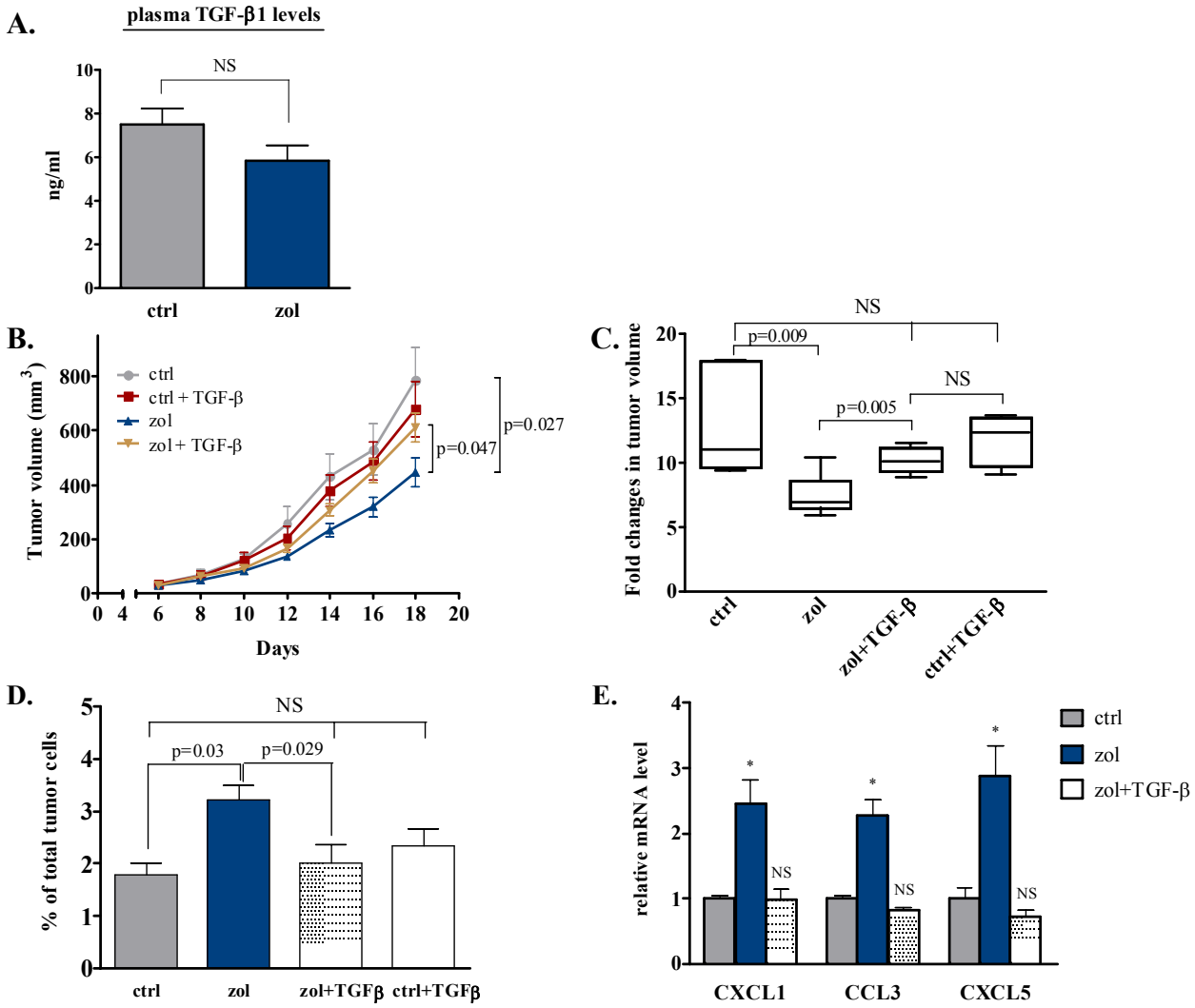


Figure 3.11: Systemic rTGF-β administration abrogates the effects of zoledronate on tumor growth and neutrophil infiltration. (A) Mean plasma TGF-β1 levels in LLC tumor bearing untreated and treated mice (n=8/group). Blood was collected 24 hours after the last treatment and TGF-β1 levels were measured as described in Materials and Methods. **(B)** Mice bearing LLC tumors were divided into four groups (n=8/group) and treated as follows: no treatment; rTGF-β; zoledronate only; and zoledronate in combination with rTGF-β. rTGF-β was administered i.p. three times per week at a dose of 100 ng/mouse. Comparison of mean tumor volume ±SEM administration is shown. **(C)** Fold changes in tumor volume ±SEM that was calculated as ratio of volume on treatment day 19 divided by volume on treatment day 1. **(D)** Flow cytometry was performed on digested tumors. The graph summarizes the percentage of $Ly6G^+$ cells out of all tumor cells. **(E)** TGF-β suppresses zoledronate-induced CXCL1, CCL3 and CXCL5 expression in LLC cells *in vitro*. Cultured LLC cells were either left untreated or treated with 10μM of zoledronate alone or in combination with rTGF-β (10 ng/ml) for 16 hours. Real-time PCR was performed and relative mRNA levels were calculated using the standard $2^{-\Delta\Delta ct}$ method[227]; normalized to the levels of the β-actin mRNA and reported as mean ± SEM. Statistical significance was assessed by two-sided Student's t-test.

3.9 Blocking TGF-β enhances *in vitro* migratory properties of neutrophils

To further validate the influence of TGF-β on neutrophil migration in zoledronate-treated animals, an *in vitro* migration assay was conducted using LLC cancer cell conditioned medium as chemoattractant. Neutrophils isolated from none marrow of non-tumor bearing naïve mice were utilized for testing the effect of reduced TGF-β concentrations on the migratory abilities of neutrophils. Since serum contains very high levels of TGF-β, the levels in serum of neutrophil growth media were titrated by using increasing amounts of TGF-β neutralizing antibody. After overnight incubation at varying levels of TGF-β neutralizing antibody, neutrophils were seeded in transwells in order to test their migratory capabilities towards cancer conditioned medium. Interestingly, the chemotactic migration of neutrophils increased in direct proportion to increasing amounts of TGF-β neutralizing antibody, suggesting that neutrophils under low TGF-β concentrations are more responsive to chemotactic signals. Neutrophil migration was further increased in transwells containing conditioned medium obtained from zoledronate-treated LLC cells (Figure 3.12). These results are helpful in explaining the reduced numbers of neutrophils observed in tumors treated with rTGF-β in conjunction with zoledronate, in comparison to those treated with zoledronate alone.

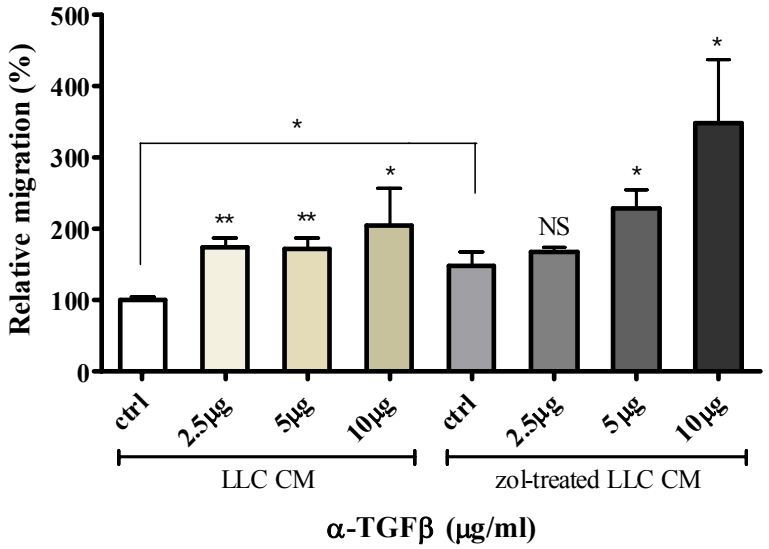


Figure 3.12: Neutrophils cultured under reduced TGF-β concentration conditions exhibited improved migratory abilities. GFP expressing neutrophils were isolated from bone marrow of MacGreen mice as described in Materials and Methods section and cultured in media (DMEM-2.5% FCS, G-CSF 100ng/ml, M-CSF 5ng/ml) supplemented with 2.5, 5 or 10 µg/ml anti-TGF-β antibody or its isotype control for 16 hours. Cell migration was assayed using transwell chambers containing 3µM pore-size membranes. Neutrophils ($1x10^5$) previously treated with different concentrations of TGF-β inhibitor antibody were seeded to the upper chamber. The lower chamber was filled with conditioned medium from LLC cells treated without (LLC CM) or with zoledronate (zol-treated LLC CM) at a concentration of 10 µM. After 4 hours, percentage of migrating cells was scored by measuring the intrinsic GFP signals emitted by neutrophils on the undersurface of the polycarbonate membranes. The percentage of migrating cells was determined relative to the control (isotype Ab treated neutrophils). Data are the means ± SEM of three independent experiments. Statistical significance was assessed by two-sided Student's t-test.

3.10 Antitumor effects of zoledronate is augmented by liposomal encapsulation

As shown in the initial studies described above, zoledronate demonstrated a statistically significant but modest *in vivo* antitumor effect in the chosen tumor models. The limited antitumor efficacy of zoledronate *in vivo* could be due to its rapid clearance from the blood as well as its high and preferential localization in bone. For these reasons, therapeutically effective serum levels of zoledronate may be difficult to achieve. Therefore, the anti-cancer activities of zoledronate may be inadequate in cases of cancers occuring outside of bone tissues. In an attempt to increase the plasma half-life and to improve tumor delivery of the drug, we encapsulated zoledronate in liposomes. Antitumor activity of

liposome encapsulated zoledronate (zoledrolip) on LLC tumors was evaluated in comparison to free zoledronate and empty liposomes that were used as an additional control for zoledrolip. LLC tumor-bearing mice were treated with comparable amounts of zoledronate in either free form or as zoledrolip. It is important to note that none of these agents was found to cause any significant changes in body weight, toxic adverse events or animal deaths. After five individual treatments, resulting in a total dose of 15 μg zoledronate per mouse the average tumor sizes in the free zoledronate treated group was reduced by 40% compared to those in the untreated control group. On the other hand, in zoledrolip treated animals, average tumor size was 55% smaller compared to standart untreated control group, and 40% smaller than in the control group receiving empty liposomes (Figure 3.13A). Similar reductions in tumor size were achieved in MC38 tumor-bearing mice upon zoledrolip treatment (Figure 3.13B). Liposomal encapsulation of zoledronate significantly improved the tumor growth-inhibiting effect of the drug. Notably, administration of empty liposomes also inhibited tumor growth significantly. It is known that plain liposomes can instigate a transient inflammatory reaction, thereby causing a partial reduction of tumor growth. However, use of liposomes alone did not account for the improvement in growth-inhibiting effect observed for zoledrolip compared to free zoledronate.

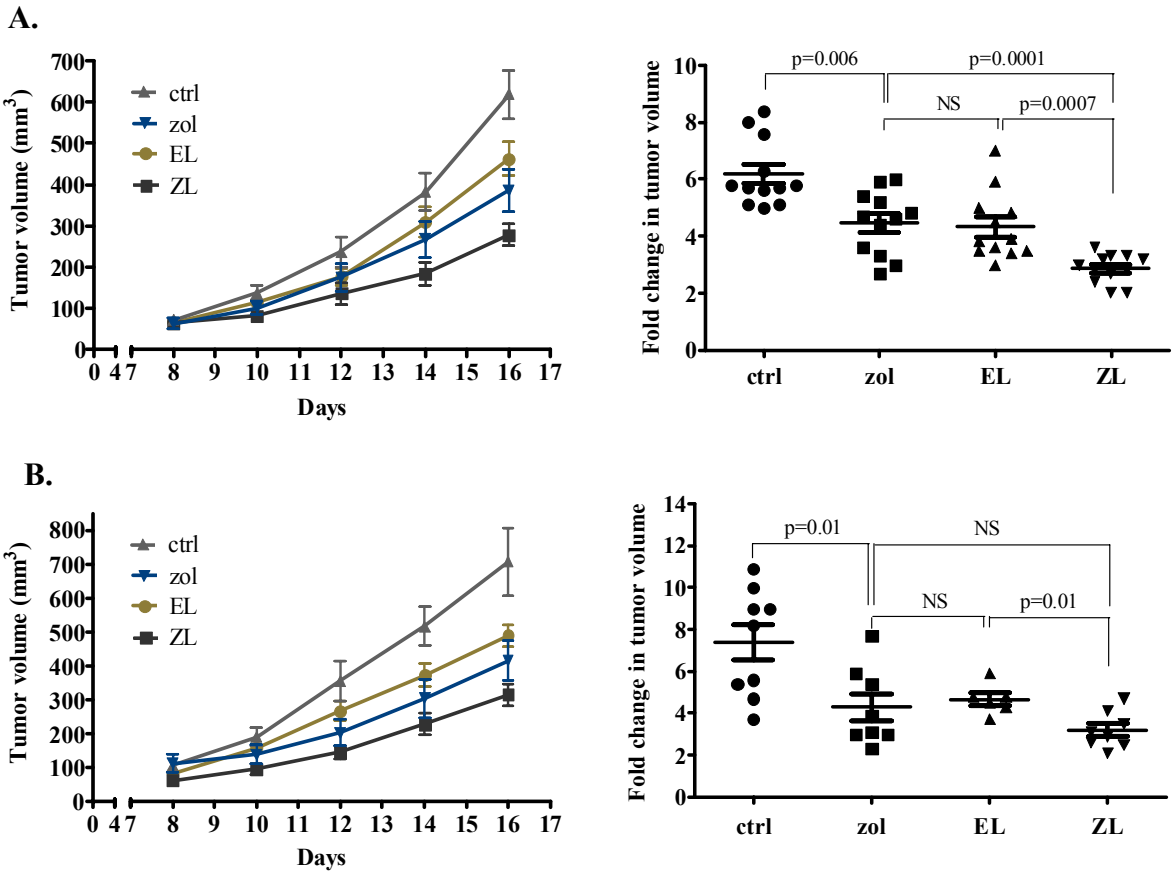
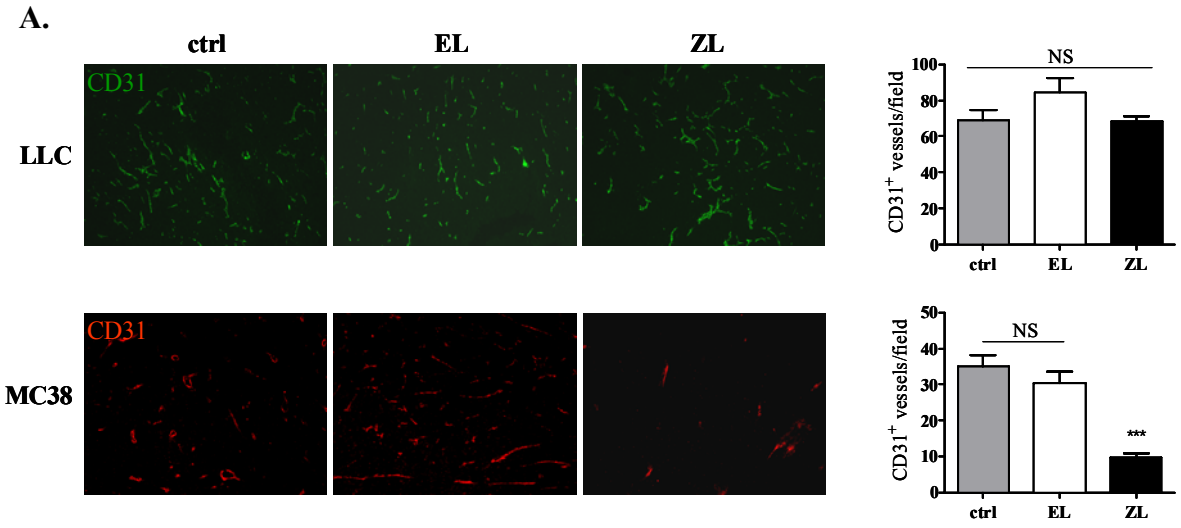


Figure 3.13: Antitumor activity of liposome encapsulated zoledronate on LLC and MC38 tumors. Female C57/BL6 mice were s.c. inoculated with LLC or MC38 cells ($5x10^5$) suspended in 50 µl HBSS. When tumors had reached a volume of 50 mm^3, animals (n=6-8 per group) were treated with zoledronate (3µg i.p. per mouse) or with the same concentration of zoledrolip (ZL) (2.4 µl of 1.25 mg/ml zoledronate containing liposomes) or empty liposomes (EL) as controls starting at day 7 and given every other day until sacrifice. Tumor size was measured every second day. **(A)** LLC tumor growth expressed as mean tumor volumes ±SEM (left panel) and as the fold changes of tumor volume relative to the first measurement at day 7 (right panel). **(B)** Growth of MC38 tumors expressed as mean tumor volumes ±SEM. Data were pooled from two independent experiments (n=6-8 mice per group).

To determine whether the similar mechanisms of free zoledronate mediated growth reduction apply to zoledrolip, animals treated with zoledrolip were examined for the previously observed effects of the free drug. Therefore we first tested the potential anti-angiogenic properties of zoledrolip in the LLC and MC38 tumor models which differ in their sensitivity towards anti-angiogenic drugs. Briefly, MC38 is known to be sensitive

whereas LLC is known to be resistant to anti-angiogenic treatments that employ an anti-VEGF antibody.[93] As expected, a divergent effect of zoledrolip was noted in the two tumor models. We found zoledrolip to be highly effective in inhibiting angiogenesis in MC-38 tumors. However, in concurrence with free zoledronate, zoledrolip did not have significant effect on angiogenesis in LLC tumors, further corroborating previous reports that LLC tumors are not affected by reduced VEGF bioavailability (Figure 3.14A). Since angiogenesis inhibition does not explain the zoledrolip-induced growth reduction in LLC tumors, we explored whether zoledrolip could also exert antitumor effects through modulation of infiltration and polarization of neutrophils, as free zoledronate does.

For this purpose, neutrophil infiltration into LLC tumors was examined in digested tumor tissue samples. In line with free zoledronate treatment, zoledrolip-treated tumors were also infiltrated with higher numbers of neutrophils in comparison to empty liposome treated tumors (Figure 3.14B). Furthermore, neutrophils isolated from zoledrolip-treated tumors exhibited a more immunostimulatory phenotype (Figure 3.14C). Accordingly, depletion of neutrophils in zoledrolip treated mice rendered zoledrolip ineffective in restricting tumor growth (Figure 3.14D). In contrast, whereas empty liposomes impaired tumor growth to a certain extent, neutrophil depletion had no observable effect on tumor growth in empty liposome-treated mice, suggesting that empty liposomes influence tumor growth by different, independent mechanisms. Although no measurable changes were detected in the plasma levels of TGF-β in zoledrolip-treated mice, rTGF-β administration in zoledrolip treated mice resulted in reversal of the zoledrolip mediated tumor growth reduction (Figure 3.14E). These results suggest that free zoledronate and zoledrolip employ similar mechanisms to impair tumor growth in LLC tumors. The enhanced anti-tumor efficacy of zoledrolip can be explained by changes in the pharmacokinetic properties, the organ distribution and tumor accumulation of the drug, compared to the free from. In this regard, the biodistribution of ^{14}C-labeled zoledronate and zoledrolip was analyzed in LLC tumor bearing mice. A significant difference was found in the distribution of zoledronate in tumors after i.v. injection of different drug forms. As shown in Figure 3.14F zoledrolip accumulated at a 10-fold higher amount in tumors as compared to free zoledronate. Furthermore, zoledrolip exhibited considerably prolonged circulation time in blood.


A.
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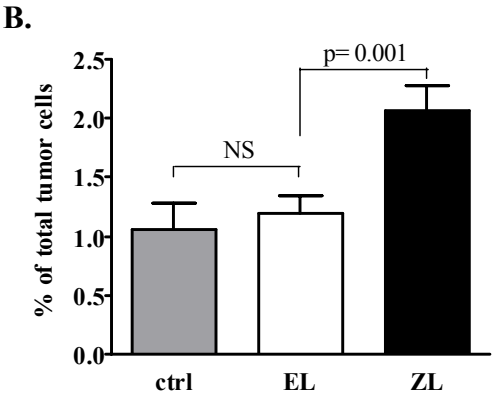
B.
% of total tumor cells
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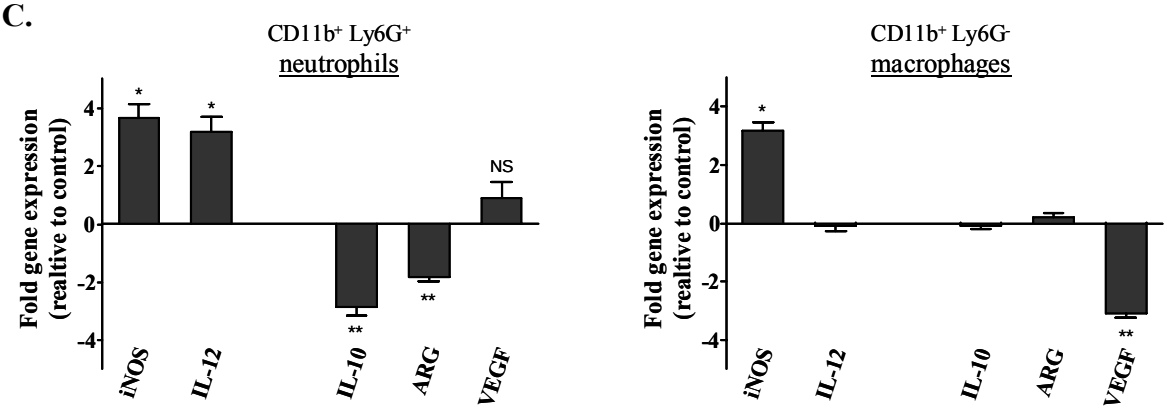
C.
CD11b+ Ly6G+
neutrophils
CD11b+ Ly6G-
macrophages
Fold gene expression
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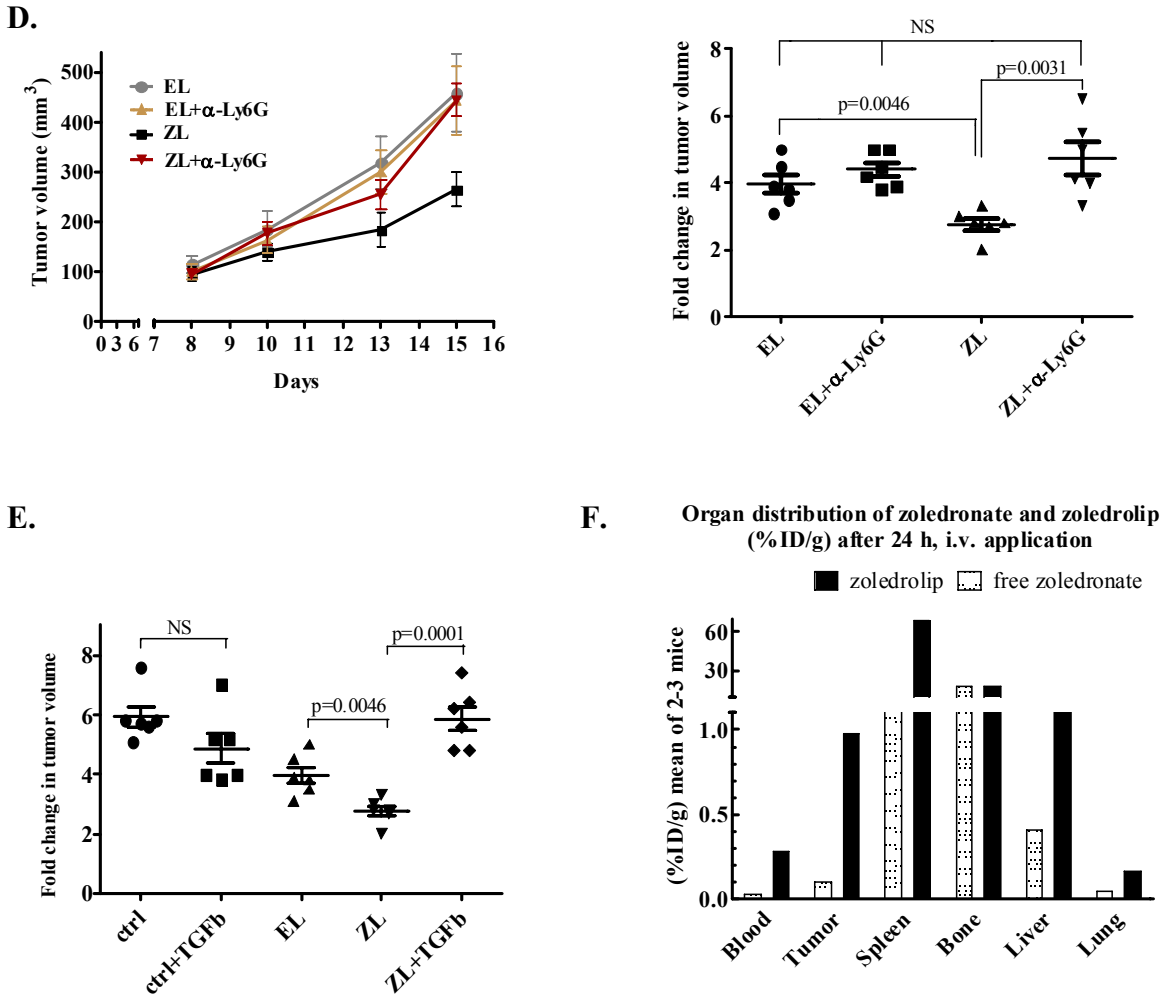

Figure 3.14:Liposomal formulation of the drug functions through similar mechanisms to delay tumor growth. (A) Tumors excised from untreated animals and animals treated with empty liposome or zoledrolip were embedded in paraffin, sectioned, and subjected to IHC. Angiogenic status was examined on LLC and MC38 sections. Representative images of CD31 IHC (left panels). Right panels depict mean numbers of vessels per field ±SEM (×100 magnification). Microvessels were counted in five randomly selected fields of tumors from four mice of each group. **(B)** Flow cytometry was performed on digested LLC tumors extracted from untreated animals and animals treated with relevant reagents. The percentage of $CD11b^+$ $Ly6G^+$ cells out of all tumor cells in all treatment groups (n=8-10 mice/group) is shown. **(C)** Neutrophils ($CD11b^+Ly6G^+$) and monocytes/macrophages ($CD11b^+Ly6G^-$) were isolated from zoledrolip treated and untreated tumors. Isolated cell populations were subjected to gene expression analysis by real-time PCR for the representative M1/M2 or N1/N2 factors. Fold change of each factor was calculated using the expression levels in untreated control cell populations as the denominator. Data are representative of 2 independent experiments. **(D)** Mice bearing LLC tumors were divided into four groups (n=6/ group), and treated as follows: empty liposomes; empty liposomes in combination with anti-LyG Ab (1A8); zoledrolip; and zoledrolip in combination with anti-Ly6G Ab. Animals were treated as described in the legend of Figure 3.10. Graphs depict the differences in mean tumor size ± SEM

(left panel) and tumor size of individual mice (right panel) from each group. Data are representative of 2 independent experiments. **(E)** Mice bearing LLC tumors were divided into four groups (6 mice/group), either no treatment or treated as follows; administration of rTGF-β; zoledronate only; and zoledronate in combination with rTGF-β. Treatments were done as described in the legend to Figure 3.11. Tumor growth rates of LLC tumor bearing individual animals is presented as fold changes of tumor volume against the first measurement at day 7. The significance was determined by two-sided student's *t*-test. (*$p<0.05$, **$p<0.01$) **(F)** LLC tumor bearing mice were administrated with ^{14}C-labeled zoledronate or zoledrolip intravenously. Animals were euthanized 24 h after i.v. administration; organs were removed, digested and processed as described in Materials and Methods (unpublished results R. Schwendener).

4. DISCUSSION AND OUTLOOK

The therapeutic efficacy of a cancer drug is heavily dependent upon the tumor microenvironment. In most cases, the tumor microenvironment is largely influenced by myeloid cells, in particular neutrophils and macrophages, which are known to augment tumor growth and also to confer drug resistance.[36] Therefore, modulation of myeloid cell properties can be valuable in restricting malignant tumor growth as well as in improving the therapeutic efficacy of conventional drugs. This study has presented the novel *in vivo* immunomodulatory property of zoledronate. We demonstrated that the drug restricts tumor growth by modulating the anticancer immune response. Our major finding is the ability of zoledronate to skew neutrophil polarization from the immunosuppressive pro-tumorigenic N2-like phenotype to the immunostimulatory anti-tumorigenic N1-like phenotype.

4.1 In vivo antitumor activity of zoledronate in syngeneic mouse tumor models

Due to its osteoclast inhibitory activity, zoledronate has become widely used in oncology for the treatment and prevention of cancer-related bone diseases as well as bone metastases.[208] To date, zoledronate research in cancer has largely focused on its effects on primary tumors located in bone and on the formation of bone metastases. Adjuvant zoledronate treatment has been reported to improve the 1-year survival rate for breast, prostate and bladder cancers which are the most common primary cancers that metastasize to the bones.[209,228,229] Prolonged survival of zoledronate-treated patients was generally attributed to a reduction in bone metastasis, a result of the anti-bone resorption properties of the drug. Whether clinically relevant doses of zoledronate can exert antitumor activities on primary tumors and metastases occurring in tissues other than bone is not conclusively understood at present.

There are only a few recent studies describing the effects of zoledronate on growth of soft tissue tumors.[176,230-233] In these animal studies, zoledronate was generally used at doses higher than recommended in the clinical practice. Thus, it remains important to determine if a clinically relevant dosing regimen of zoledronate can achieve meaningful antitumor effects in animal models of soft tissue tumors. In this study, we treated female

immunocompetent C57BL/6 mice bearing syngeneic subcutaneous tumors with zoledronate, examining whether the drug has an impact on the growth of primary tumors in tissue other than bone. The zoledronate dosage given (150 μg/kg every second day for 2 weeks) was adapted from pharmacokinetic data of cancer patients receiving the equivalent standard clinical dose.[210] Although zoledronate is administered intravenously on a monthly regimen in humans, several studies suggested that it is best supplied on a daily regimen in mice, reflecting differences in pharmacokinetics.[176] Our results show that zoledronate at doses equivalent to those approved for clinical use significantly reduces the growth of LLC and MC38 tumors but not B16 tumors.

Seeking to investigate the mechanistic basis of the reduced tumor growth, we assessed the frequency of apoptosis and found increased numbers of apoptotic cells in zoledronate treated tumors, while tumor cell proliferation itself was not affected. Zoledronate does not appear to act as an antimitotic drug. Collectively, these data suggest that the biological effects of zoledronate result from increased apoptosis in tumor cells. It is very unlikely that zoledronate has a direct effect on cancer cell survival, since cytotoxic concentrations of the drug are difficult to achieve in tumors *in vivo*. In cancer patients, following *i.v.* administration of a standard 4 mg dose of zoledronate, peak plasma levels are estimated to be 1–2 μM.[168] According to pharmacokinetic studies in mice, animals treated with zoledronate at 150μg/kg are expected to reach peak serum concentrations of about 1-3 μM.[234,235] Kuroda et al reported that 20 to 30 μM zoledronate was required *in vitro* over 24 to 48 hours to induce apoptosis in BV173 (Pre-B acute leukemia cell line) cells.[231] Other reports have shown zoledronate manifesting a direct antitumor activity *in vitro* at even higher concentrations which ranged from 50 μM to 1 mM, concentrations that are 25 to 500 times higher than those achievable *in vivo*.[211,212] Obviously, the achievable circulating concentrations of free zoledronate are too low to exert a direct pro-apoptotic effect on cancer cells.

In regard to other potential targets of zoledronate action, we tested whether the increased apoptosis in treated tumors is due to impaired angiogenesis, and indeed, anti-angiogenic properties of the compound were reported in a number of studies. Santini et al

and others showed that systemic and intratumoral VEGF levels were lowered following zoledronate administration.[236] Giraudo and colleagues reported that reduced VEGF availability upon zoledronate treatment impairs angiogenesis and tumor growth in cervical cancer.[176] Although we observed similar reductions in VEGF expression in myeloid cells isolated from zoledronate-treated tumors, the free drug was ineffective in inhibiting angiogenesis in either tumor type. However, it must be noted that our data are based on end-of-study analysis and so we cannot exclude the possibility of temporal changes in microvascular density during tumor growth which might also affect overall tumor growth rate. Furthermore, without affecting the number of vessels, it is possible that zoledronate may alter structure and function of blood vessels in tumors. These parameters should also be analyzed in further studies.

4.2 Zoledronate impairs tumor growth by interfering with neutrophil support

In regard to potential cellular targets of zoledronate action, we cannot exclude the possibility that due to their high endocytic activity myeloid cells are in a privileged position to take up zoledronate, even at low concentrations. It is intriguing that although we observed a substantial increase in the number of infiltrating neutrophils, the frequency of other myeloid cells was not appreciably altered. Notably, increases in neutrophils were found in blood, spleen and peritoneum but not in bone marrow, suggesting that this effect does not result from increased generation of neutrophils in bone marrow but rather through their increased release from bone marrow following augmented production of neutrophil chemoattractants. In this regard our data show that zoledronate increased the expression of major neutrophil chemoattractants within the tumor that may lead to increased recruitment of these cells into tumors. *In vitro* data further confirm that, zoledronate can directly induce the increased expression of these chemokines in cancer cells. On the other hand, it remains to be resolved whether the higher abundance of neutrophils in treated tumors is also associated with an increased life-span of these cells.

There is an increasing interest in elucidating the role of neutrophils in tumor progression, with various evidences implicating them in either promoting or inhibiting tumor growth and spread. Recently, the contradictory role of neutrophils in both tumor

suppression and promotion was re-evaluated in terms of the characterization of different types of tumor-associated neutrophils with polarized N1 or N2 phenotypes.[60] Referencing these studies, further phenotypic analyses of these cells revealed that neutrophils from zoledronate-treated mice exhibited an antitumor activated phenotype. Tumor-infiltrating neutrophils from untreated control animals were found to adopt an N2 phenotype that is characterized by the higher expression of the immunosuppressive factors IL-10, VEGF, TGF-β along with lower levels of immunostimulatory factors such as IL-12. Note that VEGF is a molecule exhibiting a dual profile as apart from its well-known pro-angiogenic function, it also exerts immunosuppressive effects.[237] Consistent with this N2 phenotype, depletion of the pro-tumorigenic neutrophils delayed tumor growth in untreated mice. Conversely, neutrophils from zoledronate-treated mice exhibited the opposing N1 phenotype, with higher levels of IL-12 and lower levels of IL-10 and TGF-β. Furthermore, discrimination between pro- and anti-tumorigenic activated neutrophils was also performed based on their differential L-arginine metabolism via arginase and iNOS (see Figure 3.9). Nitric oxide synthases (NOS) are important in the metabolism of L-arginine to NO, an critical mediator in inflammation and immune reactions, while arginase can convert L-arginine to urea and L-ornithine, a suppressor of cytotoxic T lymphocyte activity as well as a precursor of the polyamines spermine and spermidine that can act as tumor growth factors.[238] Transcription of iNOS is known to be increased in antitumor activated myeloid cells, resulting in prolonged high levels of NO production.[239] The iNOS enzyme has been directly implicated in macrophage-mediated tumoricidal activity and several studies have demonstrated that NO donors are cytotoxic and pro-apoptotic to tumor cells.[240] In our experiments, neutrophils from tumors of zoledronate-treated mice exhibited upregulation of iNOS, along with reduced expression of the immunosuppressive factor arginase. Therefore, we further speculate that the observed higher expression of iNOS in neutrophils isolated from zoledronate-treated tumors might be accompanied with significant tumor cytotoxicity.

Our findings are supported by a recent report claiming tumor associated macrophages as the most important immune targets of zoledronate-mediated antitumor activity.[178] In agreement with our results regarding myeloid cell polarization, these authors showed that zoledronate treatment *in vivo* induced higher iNOS expression, concomitant

with enhanced NO production in $CD11b^+$ cells isolated from the peritoneum of animals bearing mammary tumors. In addition, reduced IL-10 and enhanced IFN-γ levels were observed in the stroma of zoledronate-treated mammary tumors. [258] However, in our study we dissected the $CD11b^+$ population into $Ly6G^+$ (neutrophil) and $Ly6G^-$ (monocyte/macrophage) subsets and revealed that zoledronate induces changes in the activation status of neutrophil, but not macrophage, subsets of $CD11b^+$ myeloid cells. Although macrophages from treated tumors do exhibit upregulated expression of iNOS, they do not exhibit a fully repolarized phenotype.

Gene expression signatures reflecting a more tumor-cytotoxic N1 phenotype for neutrophils of zoledronate-treated animals suggest a potential role for these cells in zoledronate-induced tumor growth inhibition. Depletion of neutrophils in zoledronate-treated mice reversed the antitumor effect of treatment, while augmenting tumor growth in untreated mice (see Figure 3.10). These results confirm the critical role of neutrophils in zoledronate-induced antitumor activity. Additionally, the differences exhibited under zoledronate treated and untreated conditions in terms of tumor growth response to Ly6G depletion reflect the presence of different phenotypes of tumor-associated neutrophils in each condition.

Another finding that points towards the importance of neutrophils in zoledronate-induced antitumor effects is the correlation between abundance of neutrophils in tumors and the ability of the tumors to respond to zoledronate treatment. In tumors, such as LLC and MC38, with a relatively high infiltrate of neutrophils (approximately 2 % of total tumor cells), zoledronate delayed tumor growth significantly, but in tumors like B16 that lack significant neutrophil infiltration (approximately 0.2 % of total tumor cells), zoledronate had a slight but insignifiant antitumor effect.

As our findings were comparable in two different tumor types (LLC and MC38), we suggest that N1 polarization could be a general response of the tumor microenvironment following zoledronate treatment. Having illustrated the ability of zoledronate to directly or indirectly influence neutrophils to assume an antitumor phenotype, our study supports the idea that neutrophil function can be tailored *in vivo* to achieve an enhanced antitumor immune response.

These results are likely to be relevant to human cancers. A recent study by Gnant et al.[209] showed that a significant increase in disease-free survival was recorded in women with early stage breast cancer after treatment with zoledronate in combination with aromatase and tamoxifen as compared with patients treated with tamoxifen and aromatase alone. Given that neutrophil-derived factors are known to be critical in the progression and metastasis of human breast cancer, the clinical efficacy of zoledronate could be ascribed to its ability to re-polarize the neutrophils in the tumor microenvironment.[241]

4.3 TGF-β complementation reduces therapeutic efficacy of zoledronate

The identification of neutrophils as the significant anticancer cell type in zoledronate treated tumors is an exciting finding, due to the accumulating evidences regarding their tumoricidal properties which can be further enhanced by inhibition of TGF-β signaling. TGF-β is often overexpressed by tumors and plays a major role in regulating migration[242] and function[61] of neutrophils in tumors. Recent studies showed that inhibition of the TGF-β signaling pathway increases the recruitment of neutrophils in cancer and in some types of chronic disease states.[60,243] Neutrophils isolated from tumors of TGF-β receptor-blocked mice were shown to exhibit a pro-inflammatory phenotype that is strikingly similar to the phenotype of neutrophils in zoledronate-treated mice.[60] These findings correlate closely with our own observations in relation to the zoledronate-induced pro-inflammatory phenotype as well as increased recruitment of neutrophils. On the basis of these similarities between the effects of TGF-β inhibitors and zoledronate, we hypothesized that zoledronate could modulate neutrophil migration and/or function through altered TGF-β signaling. Our hypothesis is further supported by the recently reported application of bisphosphonates in alleviating skeletal-related pathologies in cancer, including bone metastasis, which coincides with a reduction in TGF-β signaling activity[200,244]. It is known that TGF-β is sequestered at high levels in bone matrix and that inflammatory cytokines, as major mediators of osteolysis, can modulate TGF-β bioavailability [245-247]. Primary tumors or those metastasized to bone can stimulate osteoclast-mediated osteolysis by increasing the levels of parathyroid hormone-like protein and RANKL [207,248,249]. The increased bioavailability of TGF-β in bone caused by osteolysis

can strongly influence the phenotype of developing neutrophils and their migratory capabilities. Moreover, we found that only a very low amount of zoledronate reaches the tumor tissue and so is unlikely to directly affect tumor cells, whereas the majority of the drug reaches the bone (see Figure 3.14G). As an effective inhibitor of osteoclast activity, zoledronate may reverse tumor-induced hyperactivity of osteoclasts, thereby normalizing serum TGF-β levels in tumor-bearing mice. In fact, it was postulated previously that bisphosphonates could have an antitumor activity by altering the release of growth factors, particularly TGF-β, in the bone microenvironment.[200] Therefore, we decided to look for paracrine mechanisms which might give rise to phenotypes associated with zoledronate treatment in mouse tumor models. Due to the lack of sufficiently sensitive methods we could not analyze the direct modulation of TGF-β or its activity by zoledronate. Nevertheless, supplementation of zoledronate or zoledrolip with TGF-β reversed the zoledronate-induced enhanced recruitment of neutrophils and concomitantly restored tumor growth to levels similar to those in untreated controls (see Figures 3.11 and 3.14). These results imply that TGF-β dominates over zoledronate activity. Our *in vitro* results further support this hypothesis by revealing that upregulated expression of neutrophil chemoattractants induced by zoledronate treatment were reduced to control levels by supplementation with recombinant TGF-β (see Figure 3.11). It is also known that increasing TGF-β levels can deactivate myeloid cells by decreasing expression of critical transcription factor genes such as NFκB and STAT1.[114] Therefore, it seems probable that the increased inflammatory activities of neutrophils in zoledronate-treated tumors are due to increased expression of these critical transcription factors.

Since zoledronate mimics the effects of TGF-β signaling inhibitors, we propose that it can provide an alternative to the need for such signaling inhibitors. This notion is supported by a recent report showing that TGF-β repressed neutrophil migration by inhibiting expression and secretion of neutrophil chemoattractants in endothelial [242] and cancer cells[63]. In addition, our *in vitro* migration experiments revealed that neutrophils sense chemotactic signals more strongly under reduced TGF-β levels. (see Figure 3.12) These results hint that TGF-β influences migration of neutrophils not only by regulating tumor-secreted chemoattractants, but also by directly influencing intrinsic migratory

abilities of these cell types. These intrinsic changes may very likely be due to modulation of cell surface receptors for chemokines or of transcriptional regulators of inflammation. It would be interesting to test whether zoledronate influences the neutrophil migration and gene expression signature by acting directly on these cells or if the observed effects are a combined action of zoledronate and/or reduced TGF-β levels.

An important point that remains to be discussed is the role of adaptive immunity, especially of cytotoxic T cells in the effects caused by zoledronate. It is known that zoledronate can enhance the cytotoxic activity of the Vγ9/Vδ2 subset of γδT cells in cancer and that activated γδT cells can be used as cell-based therapy to treat cancer patients [191,250]. However, a murine counterpart of the Vγ9/Vδ2 subset of γδT cells has so far not been identified. Previous studies did not find any evidence of γδT-cell recruitment in the peripheral blood or lymphoid organs of zoledronate-treated mice.[250] Several studies demonstrated the ability of neutrophils to stimulate $CD8^+$ T cell proliferation and activation in cancer. [134,251] This notion finds support in the immunoregulatory gene expression profile of neutrophils isolated from zoledronate-treated tumors which express high levels of IL-12 and low levels of IL-10, VEGF and arginase. Downregulation of IL-10, arginase and VEGF in neutrophils is likely to reverse immunosuppressive effects on CD8 T cells. The pro-inflammatory cytokine IL-12 induces production of IFN-γ by macrophages and NK cells, thus favoring differentiation of naïve $CD4^+$ T cells into mature Th1 cells.[252] Accordingly, our *in vitro* co-culture experiments, demonstrating enhanced proliferation and IFN-γ production of T cells which were co-cultured with $CD11b^+$ cells from tumors of zoledronate-treated mice, confirm the immunostimulatory properties of these cells and also raise the question of the possible involvement of these CD8 T cells in zoledronate-induced antitumor effects (see Figure 3.6). However, we could not detect any significant increase in the frequency of $CD8^+$ T cells in treated tumors. In fact, subcutaneous tumors have been shown to be refractory for cytotoxic T cell response, suggesting that zoledronate might be more effective in orthotopic tumor models, and gain more clinical value.[253] Nevertheless, our results show promise in modulating the immune response in a wide variety of cancers by using zoledronate as adjuvant tumor therapy. On the basis of this study, we believe that the application of zoledronate in cancer therapy holds immense value and that further

efforts should be extended to identify the correct niches, combinations, and conditions in which this drug should be given to cancer patients.

4.4 Liposomal encapsulation potentiates the antitumor effect of the zoledronate

Stronger tumor growth inhibition was achieved by use of zoledrolip, suggesting additive therapeutic effects of liposomes. This improved effect of zoledrolip may be either due to intrinsic properties of liposomes in activating inflammatory cell types and/or due to improved delivery of zoledronate via liposome. In fact we observed that empty liposomes exhibit some degree of tumor growth inhibition suggesting that liposomes can indeed induce a weak inflammatory response. Besides that, organ distribution studies done in our laboratory showed that liposomal encapsulation of zoledronate enhanced the accumulation of the drug in tumors (up to 1% of the injected dose of zoledrolip versus 0.1% of free zoledronate injection; unpublished results R.Schwendener; see Figure 3.14G) as well as in spleen and liver. The improved distribution of liposomal zoledronate into tumor tissues can be attributed to the prolonged circulation times and passive tumor targeting properties of liposomes.[254] Previous studies have demonstrated that liposomes can extravasate through the fenestrated blood vessels that are typical of tumor neo-angiogenesis, and therefore accumulate at high concentrations in solid tumors.[255] However, as spleen, liver and tumor have abundant phagocytic cell types, the increased drug accumulation may also reflect that liposomes are directing zoledronate to myeloid cell types in these tissues. Improved delivery of zoledronate to myeloid cells by liposomal formulations might result in a more pronounced antitumor phenotype that is responsible for stronger growth inhibition effects.

Although free drug was ineffective at inhibiting angiogenesis in either tumor type, zoledrolip inhibited angiogenesis strongly in MC38 tumors but not in LLC tumors. This discrepancy in the responses of the tumor models to liposomal zoledronate corroborates with the previous reports that LLC tumors are not affected by reduced VEGF bioavailability. It is possible that redundancy of pro-angiogenic factors in LLC tumors can bypass the depletion of VEGF, perhaps through upregulation of other angiogenic factors such as FGF or angiopoietin-1 by tumor and stromal cells. In this respect, the use of the LLC tumor model, refractory to angiogenesis inhibitors, serves an important purpose in

dissecting novel mechanisms, independent from angiogenesis inhibition, that contribute to the therapeutic properties of zoledronate.

4.5 Conclusion and Outlooks

In conclusion, we showed that zoledronate, a relatively non-toxic drug currently marketed mainly for the treatment of osteoporosis and cancer-related skeletal events, is effective against experimental lung and colon carcinoma tumors. As summarized in Figure 4.1, we proposed that the drug impairs tumor growth by modulating the tumor microenvironment as shown by an increased numbers of neutrophils and their reverted polarization from the N2 to N1 phenotype. Having identified neutrophils as cellular targets of the antitumor action of zoledronate, we contributed to the body of evidence that neutrophils and their secreted products are functionally important for the growth of a variety of tumors. We further provide evidence that the use of exogenous modifiers to achieve re-orientation of these cells in favor of a more antitumoral phenotype is a promising anticancer strategy. Such an approach may improve the outcome of current immunotherapeutic strategies, which are counteracted by the potent immunosuppressive reactions in the tumor stroma.

In this study, we highlight the TGF-β signaling pathway as a potential regulatory switch for the distinct migratory and phenotypic characteristics of neutrophils in zoledronate-treated animals. Further research is warranted to fully elucidate the role of TGF-β signaling in this process. In this context, establishment and implementation of *in vivo* reporter systems would help to detect zoledronate-induced transient changes in TGF-β signaling in neutrophils. Another potential underlying mechanism of this cytokine shift might be via STAT signaling, since a recent study in mice showed that amino-bisphosphonates may prolong phosphorylation of STAT1, a key molecule in the polarization of macrophages towards an M1 phenotype.[127] Further analysis of the molecular basis of the zoledronate-induced neutrophil phenotype will help to determine other potential signaling pathways responding to zoledronate. Identification of these pathways may allow us to selectively modulate the functional activities of neutrophils in cancer.

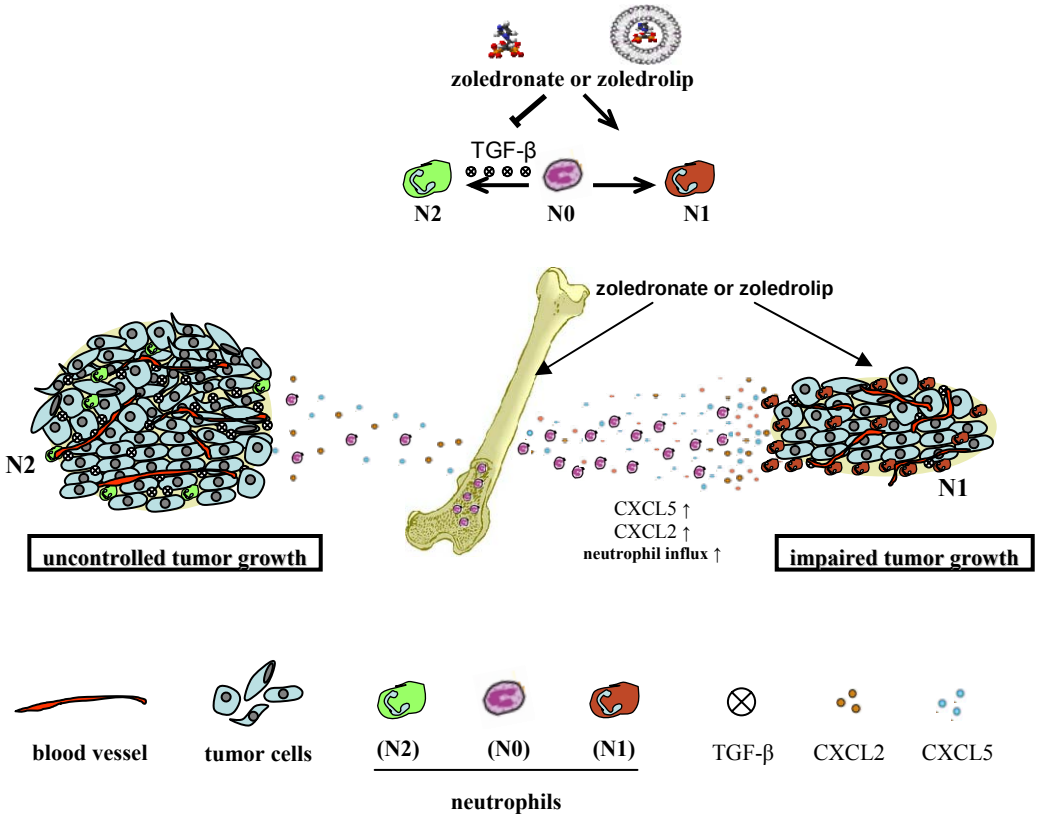

Figure 4.1: A schematic diagram showing the hypothetical antitumor mechanism of zoledronate and zoledrolip. Upon *in vivo* administration, zoledronate induces enhanced expression of neutrophil-attracting chemokines resulting in increased accumulation of neutrophils in tumors. In the tumor milieu, zoledronate skew neutrophil polarization away from their protumorigenic /immunosuppressive N2-like phenotype that is known to be regulated by TGF-β. Accordingly, TGF-β administration in treated animals reverse the anti-tumor effects of treatment. Free zoledronate primarily accumulates in bones while liposomal encapsulation of the drug improves its bioavailability in extraskeletal tumor sites that leads to stronger inhibition of tumor growth.

Although the findings of this investigation are novel and important, some limitations should be addressed. Here we explored the effect of the drug on primary tumor growth. However, effects of drugs on primary tumor growth do not necessarily predict therapeutic outcome. Cancer-related deaths are caused principally by metastasis arising from residual disease, whose therapeutic responses have been suggested to be substantially different from those of primary tumors. Future studies will be required to determine the specific contribution of zoledronate-induced activation of neutrophil functions in the migratory, invasive and metastatic features of tumor cells. As subcutaneously implanted

tumors rarely express invasive or metastatic phenotypes, these features cannot be evaluated in such models. This issue could be overcome by studying these aspects in orthotopic or spontaneously growing tumor models.

The promising antitumor efficacy of zoledrolip warrants further evaluation in combination therapy settings where zoledrolip is incorporated into conventional therapeutic regimens. In this respect, strategies encouraging the development of intense intratumoral neutrophil infiltrates may help to further improve the antitumor effect of zoledrolip. Recombinant G-CSF, a key regulator of neutrophil survival and production, is commonly used in clinics to accelerate recovery of neutrophil numbers in patients with chemotherapy-induced neutropenia.[256] However, G-CSF has recently been shown to diminish the anti-tumor activity of chemotherapy, partially due to augmented angiogenesis induced by neutrophils, indicating pro-tumorigenic activation of these cells in tumors. In relation to this point, it is possible that addition of zoledronate or zoledrolip, when conventional chemotherapy is used in combination with G-CSF therapy, might alter that outcome by polarizing G-CSF-stimulated neutrophils to elicit antitumor activity. Given the good safety profile affirmed by its common use by postmenopausal women along with the growing appreciation of its therapeutic value, zoledronate holds great promise as an immunotherapeutic adjuvant in cancer therapy.

5. MATERIAL AND METHODS

Antibodies and reagents

All the anti-mouse antibodies used for flow cytometric analyses including APC conjugated Ly6G (1A8 clone), F4/80, CD8, Pacific Blue conjugated CD11b, CD11c, CD3, PE/Cy7 conjugated CD45 and IFN-γ were purchased from Biolegend (San Diego, CA). Primary antibodies for IHC analysis of frozen and paraffin sections were a goat polyclonal CD31 (Biolegend), a rabbit monoclonal cleaved caspase-3 (Cell Signaling Technology), a rabbit monoclonal phospho-histone H3 (Cell Signaling Technology), and a biotinylated anti-GFP (GeneTex). Secondary antibodies used were donkey-anti-rat-Cy5, bovine-anti-goat-FITC, donkey-anti-rabbit-Cy5, and streptavidin Cy-5 (Jackson ImmunoResearch). The 1A8 antibody (BioXCells) was used for the *in vivo* depletion of $Ly6G^+$ cells and the rat IgG2a (2A3) antibody (BioXCell) was used as isotype control. Recombinant TGF-β1 (rTGF-β) was purchased from R&D Biosystems. Fluorescent dye CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester) was purchased from Invitrogen (Carlsbad, CA, USA). Zoledronate was kindly provided by Novartis Pharma AG (Basel, Switzerland).

Cells and animals

The LL/2 Lewis lung carcinoma (LLC1) cell line was purchased from the European Collection of Cell Cultures (ECACC catalog no. 90020104), the B16 melanoma cell line (ATCC no. CRL-6323) was from American Type Culture Collection (ATTC; Rockville, MD). The MC38 colon adenocarcinoma cell line was kindly provided by Dr. L. Borsig, Institute of Physiology, University of Zurich, Switzerland. All cell lines were of C57BL/6 mouse origin and were free of *Mycoplasma* and maintained in DMEM (Gibco) supplemented with 10% (v/v) heat-inactivated FCS (Gibco) and 0.8 % penicillin/streptomycin (Sigma-Aldrich). Cells were grown in monolayers at 37 ºC in a humidified CO_2 incubator.

All experiments were done using 8- to 12- week- old female immunocompetent mice. Wild-type C57BL/6 mice were from Charles River Laboratories (Germany) and transgenic mice expressing EGFP under the control of the CSF-1R (*c-fms*) promoter,

referred to as MacGreen mice, were provided by Dr. D.A. Hume (Edinburgh, UK).[213] Mice were kept under standard housing and dietary conditions at the University of Zurich animal facility. Animal studies were performed under license 215/2008 issued to R. Schwendener by the Veterinary Department of the Canton Zurich, Switzerland.

Murine tumor models and treatments

The syngeneic B16 melanoma, Lewis lung carcinoma and MC38 colon carcinoma tumor models were established by subcutaneous injection of 3×10^5 MC38 or B16 or 5×10^5 LLC cells suspended in 50 µl HBSS into the right hind flank of C57BL/6 wild type or MacGreen mice. Tumors were allowed to grow for 5-8 days to reach a volume of approximately 50-70 mm^3 before initiation of treatment. Once tumors had reached appropriate sizes, mice were randomly divided into treatment and control groups. The mice of treatment groups received the drugs by intraperitoneal injection as described in the legends of the corresponding figures (see Fig 3.1,2 and Fig 3.13). Control groups received an equal volume of HBSS or empty liposomes *i.p*. Treatment was repeated every 2nd day during the course of tumor growth. Tumor sizes were measured by a caliper every 2 to 3 days and recorded as tumor volume calculated by the formula $V=\pi LW^2/6$ (L: largest tumor diameter, W: perpendicular diameter). Growth was monitored until a tumor volume of 2 cm^3 was reached where after mice were sacrificed.

***In vivo* depletion of Ly6G$^+$ neutrophils**

For depletion of neutrophils, each mouse received 100 µg of anti-Ly6G mAb (clone 1A8) *i.p*. in 100 µl HBSS. Control animals were injected with 100 µg of an isotype-matched IgG2a Ab (2A3) *i.p*. in 100 µl HBSS/mouse. The dosages of the depletion and control Ab were chosen according to accepted use in the literature.[60] Injections were started 1 day before drug administration and applied repeatedly throughout the entire experimental period as shown in Fig. 3.10. Depletion was controlled by testing blood and tumor samples from treated mice by flow cytometric analysis.

Flow cytometric analysis of tumors, blood, spleen, peritoneal cells and bone marrow

At the end of the treatment period tumor tissue, spleen, peritoneal lavage cells and blood samples were collected. Harvested tumors were minced and digested with a mixture of 2 mg/ml collagenase type IV, 2 mg/ml DNase I and 500 U/mg hyaluronidase (all from Sigma) at 37°C for 30 min. Dissociated tissues were passed through a cell strainer (70 µm mesh size). Dead cells and debris were removed using the dead cell removal kit (Miltenyi Biotech). Bone marrow cells were harvested from both femurs and tibias by flushing the bone cavity with RPMI using 25 gauge needles followed by centrifugation and resuspension in PBS.

Harvested spleens were cut into 3 pieces. Splenocyte suspensions were prepared by gently meshing spleen pieces with the rubber end of a syringe plunger through the 70 µm mesh size strainer. Peritoneal cells were obtained by gently washing the peritoneal cavity with 10 ml cold PBS, then centrifuged and resuspended in PBS. Blood samples were collected in heparinized tubes (BD, Microtainer) directly from the heart by cardiac puncture.

To eliminate red blood cells in tumors, blood, bone marrow and spleen, cell suspensions were centrifuged and the pellets resuspended in FACS lysing solution (BD Pharm Lyse) and incubated for 10 min at room temperature. Then cells were centrifuged at 300 *g* for 5 min and washed two times with PBS containing 2% fetal bovine serum and 0.05% sodium azide (FACS buffer). To prevent nonspecific binding, single cell suspensions were preincubated with Fc Blocker (anti-mouse CD16/32, BioLegend) for 10 min on ice. Cells were then labeled with conjugated antibodies as listed above at 4 °C for 30 min in the dark. Cell suspensions were centrifuged at 300 *g* for 5 min and washed with FACS buffer to remove residual antibodies.

For intracellular IFN-γ staining, cells (10^6 cells/ml) were incubated in RPMI with 5% FCS for 5 hours in the presence of Brefeldin A, ionomycin and PMA to stimulate the production of cytokines and to inhibit the secretion of the synthesized cytokines before addition of Fc blocker, fixation, and permeabilization.

All experiments were performed using a CyAn 9 ADP flow cytometer (Beckman-Coulter). Data analysis was done using FlowJo software (Tree Star, Ashland, OR).

Administration of exogenous TGF-β1

Intraperitoneal injections of recombinant TGF-β1 at a dose of 1μg /mouse were started 2 days after the first zoledronate treatment and repeated two times per week during the course of tumor growth.

Isolation of $Ly6G^+$, $CD11b^+$ and GFP^+ cells

Twenty four hours after the last treatment, animals were euthanized and tumors were harvested. To isolate Ly6G and CD11b cells, tumors were digested and single cell suspensions were prepared as described. $CD11b^+Ly6G^+$ and $CD11b^+Ly6G^-$ cells were sorted using FACS Aria cell sorter (BD Bioscience). CD8 T cells were isolated from spleens of naïve mice by using the CD8a T cell isolation kit with LS columns (Miltenyi Biotec) following the manufacturer's instructions. Purity greater than 90% was deemed acceptable. $EGFP^+$ cells were isolated from tumors and peritoneal lavage using flow cytometry. Purity of the obtained cells was checked after sorting by reanalyzing the relevant fraction on the same instrument after a full cleaning protocol.

Isolation of Bone Marrow Neutrophils

Mouse neutrophils were isolated from the bone marrow of naïve mice. $Ly6G^+$ cells were isolated by using an APC conjugated anti-Ly6G antibody (clone 1A8) and anti-APC microbeads with MiniMACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. The murine neutrophil isolation protocol routinely yields cell suspensions that are >85% neutrophils. All of the neutrophil studies were carried out at 37 °C.

Immunohistochemistry

Animals bearing flank tumors were euthanized and the tumors were immediately placed in Tissue-Tek OCT compound (Sakura Finetek Inc., Torrance, CA) to be stored at −80°C, followed by sectioning. Frozen sections (8 μm) were dried in air, hydrated with PBS, blocked with 1 % BSA and anti CD16/32 Fc blocker (1:500 v/v) in PBS (containing 0.1 % Triton X-100) for 30 min followed by incubation with primary antibodies for 1 hr at room temperature (RT). Sections were washed three times in PBS followed by incubation with corresponding secondary antibodies for 1 hr. After washing in PBS, sections were treated with 4′, 6-diamidino-2-phenylindole (DAPI) for 10 min to stain nuclei, washed twice and mounted with anti-fade reagent (Thermo Scientific).

To prepare paraffin tissue sections, tumor tissues were immersion-fixed in 4% PBS-buffered formalin, embedded in paraffin, sectioned, and stained with H&E using standard techniques.

Quantitative analysis of vessels was done using the ImageJ Software (http://rsbweb.nih.gov/ij/). For each sample, CD-31-positive microvessels were counted in five x100 microscopic fields randomly selected within viable tumor areas.

***In situ* analysis of tumor proliferation and apoptosis**

To determine the proliferative and apoptotic states of tumors, phosphohistone H3 and caspase-3 staining, respectively, was performed on paraffin-embedded tumor sections. The number of apoptotic or proliferative cells in a given field (0.8×0.6 mm) was counted for quantitative analysis. Several discrete sections were examined for each tumor. Tumors from five different animals were examined for each experimental group of mice. Quantitative analysis of apoptotic and mitotic cells were done using the ImageJ software.

Immunoassay for TGF-β

The concentrations of TGF-β1 in peripheral blood plasma of treated, untreated and naïve (non-tumor bearing) mice were measured using an ELISA kit, employing the quantitative sandwich enzyme immunoassay technique (Quantikine, R&D Systems)

Briefly, blood samples were collected in heparin coated collection tubes (BD, Microtainer) by cardiac puncture at the time of euthanasia. To extract plasma, blood samples were centrifuged for 20 min at 1000 x g. Plasma was collected from supernatants and centrifuged at 10.000 x g for 10 min for complete removal of platelets. Plasma samples were assayed for TGF-β1 by following the manufacturer's instructions. The absorbance was recorded using a SPECTRAmax GEMINI XS microplate reader (Molecular Devices) at 450-nm wavelength. The ELISA for TGF-β1 included acidification and neutralization steps resulting in measurement of total TGF-β1 (both active and latent forms).

Quantitative RT-PCR

For quantitative RT-PCR, total RNA was extracted from sorted cells or tumors using RNeasy Kit (Qiagen, Valencia, CA) and reverse-transcribed with the Omniscript reverse transcriptase kit (Qiagen). Primers for TNF-α, IFN-γ, IL-12, arginase-1, CD163, TGF-β1, MHC-class II, CXCL1, CXCL5, CXCL2 and CCL3 were designed using primer design software. Predesigned, validated primer sets for IL-10 and iNOS were purchased from Origene (Rockville,MD). Real time PCR was performed on a Light Cycler 480 instrument (Roche Diagnostics) using SYBR Green PCR master mix. PCR settings were: 95ºC, 5 min, 45 cycles of 10 s 95ºC, 25 s annealing and 15 s 72ºC. The same cycling parameters were used for all primer sets. Primers were purchased from Microsynth (Switzerland) and Origene (Rockville, MD). Gene specific primer sequences and annealing temperatures are given in Table S1. Reactions were performed in triplicates for each sample. Single gene products were obtained for all reactions as assessed by melt curve analysis or gel electrophoresis. The quantity of target mRNA was normalized to GAPDH and β-Actin levels in each sample. Fold changes were calculated using the Pfaffl equation[257] and relative changes in gene expression were calculated using 2(-Delta Delta C(T)) method[227].

***In vitro* assays of CD8$^+$ T cell proliferation and activation**

Splenic CD8$^+$ T cells from naïve mice were isolated using negative selection via MACS separation, according to the CD8$^+$ T cell isolation kit II (Miltenyi Biotec). GFP$^+$ myeloid cells were isolated using FACS sorting. For the CFSE proliferation assay, splenic

$CD8^+$ T cells from naive mice were incubated for 5 min in the dark with 5 μM CFSE in PBS and then washed two times. The assay was performed in 200 μl RPMI 1640 complete medium in 96-well round bottom plates. A total of $3x10^4$ GFP^+ cells were cultured with CFSE-labeled 1×10^5 $CD8^+$ T cells (ratio 1:3) and incubated at 37°C in 5% CO_2 for 72 h. *In vitro* IFN-γ production and CFSE proliferation of $CD8^+$ T cells were measured by using CyAn ADP flow cytometer and analyzed with FlowJo Software (Tree Star).

In vitro migration assay

GFP expressing neutrophils were isolated from bone marrow of MacGreen mice by using APC conjugated anti-Ly6G antibody and subsequent anti-APC microbeads with MidiMACS LS columns (Miltenyi Biotec, Germany) following the manufacturer's instructions. Sort-purified cells were then cultured in media (DMEM-2.5%FCS, G-CSF 100ng/ml, M-CSF 5ng/ml) supplemented with 2.5 or 5 μg/ml anti-TGF-β antibody (clone 1D11, R&D) or its isotype control for 16 hours. These cells were then collected, washed and tested for migration ability.

Cell migration was assayed using Fluoroblok Transwell chambers containing 3μM pore-size membranes in 24 well plates (BD biosceinces). The lower chamber was filled with 700 μl conditioned medium from LLC cells treated with or without zoledronate at concentration of 10 μM. Neutrophils ($1x10^5$) previously treated with different concentrations of TGF-β inhibitor antibody were suspended with 250 μl medium (DMEM-2.5%FCS) seeded to the upper chamber. After 4 hours, percentage of migrating cells was scored by measuring the intrinsic GFP signals emitted by neutrophils on the undersurface of the polycarbonate membranes.

Preparation of zoledronate liposomes

Liposomes composed of soy phosphatidylcholine (SPC, 100 mg/ml), cholesterol (10 mg/ml) and D,L-α-tocopherol (0.2 mg/ml) corresponding to 1:0.2:0.01 mol parts were prepared by freeze-thawing and filter extrusion. The dry lipid mixture was solubilized in a physiologic phosphate buffer (20 mM, pH 7.4) supplemented with mannitol (230 mM, PB-Man) as cryoprotectant, allowing freezing of the liposomes, and 4 mg/ml zoledronate (2-(imidazol-1-yl)-hydroxy-ethylidene-1,1-bisphosphonic acid disodium salt, 4.75 hydrate,

$C_5H_{10}N_2O_7P_2$ 4.75 H_2O, molecular weight: 401.6, Novartis Pharma AG, Switzerland). The resulting multilamellar vesicles were freeze-thawed in 3 cycles of liquid nitrogen and water at 40ºC, followed by repetitive (5-10x) filter extrusion through 400 nm and 100 nm membranes (Nuclepore, Sterico, Switzerland) using a Lipex extruder (Lipex Biomembranes, Inc., Canada). Non-encapsulated zoledronate was removed by dialysis (Spectrapore tube, 12-14.000 mol. Wt. cut-off) with PB-Man as dialysis buffer (1:100 v/v). All preparations were sterile filtered through 0.45 μm filters (Pall Gelman Laboratory) and aliquots of 0.05 to 0.1 ml stored at -80ºC. Liposomes containing ^{14}C-labelled zoledronate (specific activity 7.07 MBq/mg, obtained from Novartis) were prepared accordingly by trace labeling. Liposome size and homogeneity were routinely measured with a Nicomp laser light scattering particle sizer (Nicomp 370, Sta. Barbara, CA). Zoledronate liposomes contain approximately 1.25 mg/ml zoledronate. Control liposomes were prepared accordingly, except for the addition of zoledronate. Both types of liposome formulations have a mean diameter of 135±70 nm.

Determination of ^{14}C-zoledronate distribution in LLC tumor bearing mice

Organ and tumor distribution was essentially done as described by Marty et al.[258] Mice received 3 x 10^5 LLC cells s.c. on both sides of the flanks. As tumors had reached sizes of approximately 0.5 cm in diameter, radiolabeled free zoledronate or zoledronate liposomes were injected i.v. After 24 h the animals were anaesthetized and sacrificed by heart puncture and blood, lung, liver, spleen, kidneys, bone (femur and tibia) and tumors were removed, weighed, solubilized in Soluene-350 (Perkin Elmer, Shelton, CT, USA) and the radioactivity measured by scintillation counting using Ultima Gold XR (Perkin Elmer) liquid scintillation cocktail with a TriCarb liquid scintillation system (PerkinElmer) Blood correction factors were applied to all organ samples.[259] Experiments were done by R. Schwendener.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.02 software (GraphPad Software, Inc.). Values are expressed as mean ± SEM. Statistical comparisons were made using the two-tailed Student's *t*-test for single value comparisons or one-sample *t*-test and

2-way ANOVA for comparisons of 3 or more groups. Differences were considered to be statistically significant when $p<0.05$.

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7. LIST of ABBREVIATIONS

Ab: Antibody
ANT: Adenine nucleotide translocase
ATP: Adenosine triphosphate
bFGF: Basic fibroblast growth factor
BMP: Bone morphogenic protein
CCL2: Chemokine C-C motif ligand 2
CIS: Carcinoma in situ
CML: Chronic myeloid leukemia
COX-2: Cyclo-oxygenase 2
CSF-1: Colony stimulating factor
CSFE: 5,6-carboxyfluorescein diacetate succinimidyl ester
GGPP: Geranylgeranyl pyrophosphate
EGF: Epidermal growth factor
H&E: Haematoxylin and Eosin
FGF: Fibroblast growth factor
FPP: Farnesyl pyrophosphate
HIF-1:Hypoxia inducible factor
HRG: Histidine-rich glycoprotein
iNOS: Inducible nitric oxide synthase
IFN-γ: Interferon gamma
IHC: Immunohistochemistry
LLC: Lewis lung carcinoma
LPS: Lipo-polysaccharide
M1: Pro-inflammatory macrophages, M1 phenotype
M2: Pro-tumor macrophages, M2 phenotype
Mac 1: Macrophage antigen 1
MDSCs: Myeloid-derived suppressor cells
MCP-1: Monocyte chemotactic protein-1
MMP: Matrix metalloproteinase
MPS: Mononuclear phagocytic system
MVD: Mean vascular density
N1: Pro-inflammatory neutrophils, N1 phenotype
N2: Pro-tumor neutrophils, N2 phenotype
N-BPs: Nitrogen-containing bisphosphonates
NFκb: Nuclear factor-κb
PTHrP: Parathyroid hormone-related peptide
PDGF: Platelet derived growth factor
PlGF: Placenta growth factor-1
PPi: Pyrophosphate analogs
PyMT: Polyoma middle-T-oncogene
RANKL: Receptor activator of nuclear factor kappa-B ligand
RES: Reticuloendothelial system
ROS: Reactive oxygen species
rTGF-β: Recombinant transforming growth factor-beta

SDF-1: Stromal derived factor-1
SHIP1: SH2 (Src homology 2)-containing inositol phosphatase-1
SRE: Skeletal related events
STAT: Signal transducers and activators of transcription
TAM: Tumor associated macrophage
TGF-β: Transforming growth factor-beta
TILs: Tumor infiltrating leukocytes
TNF-ά: Tumor necrosis factor-alpha
T_{regs}: T-regulator cells
uPA: Urokinase-type plasminogen activator
VEGF: Vascular endothelial growth factor
VEGFR1: Vascular endothelial growth factor receptor 1
Zol: Zoledronate (administered in the free form)
Zoledrolip: Liposome encapsulated zoledronate

8. APPENDIX

Supplementary Figure S1.

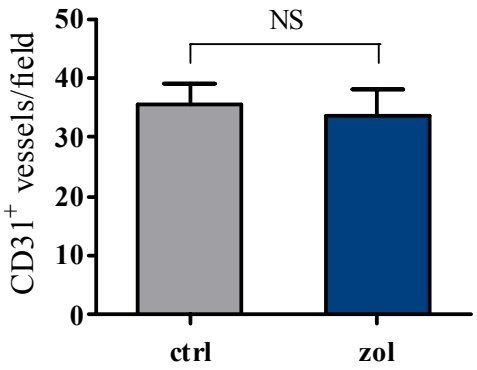

Figure S1:Effect of zoledronate on MC38 tumor angiogenesis. MC38 tumors excised from zoledronate treated and untreated animals were embedded in paraffin, sectioned, and subjected to immunofluorescent staining with anti-CD31.Diagrams depict mean numbers of vessels per field ±SEM (×100 magnification). Microvessels were counted in five randomly selected fields of tumors from four mice of each group.

Supplementary Figure S2.

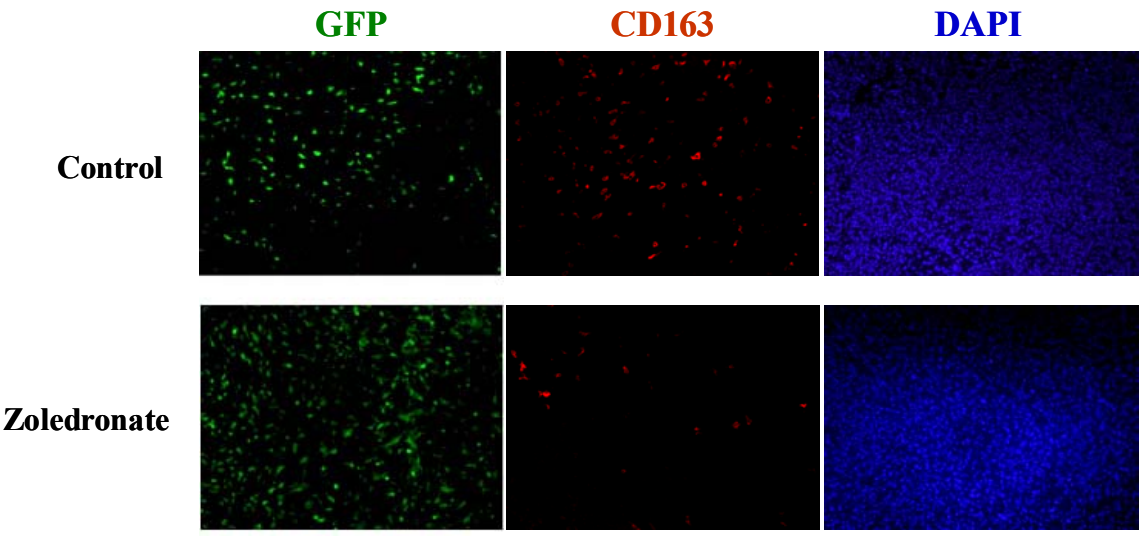

Figure S2: Infiltration of LLC tumors with myeloid cells. LLC tumors from treated and untreated MacGreen mice were removed, paraffin embedded, sectioned and stained for CD163 (red), GFP (green) and DAPI (blue). IHC analysis revealed that although the frequency of myeloid cells (green) increase, the abundance of $CD163^+$ cells decrease in treated tumors.

Supplementary Figure S3.

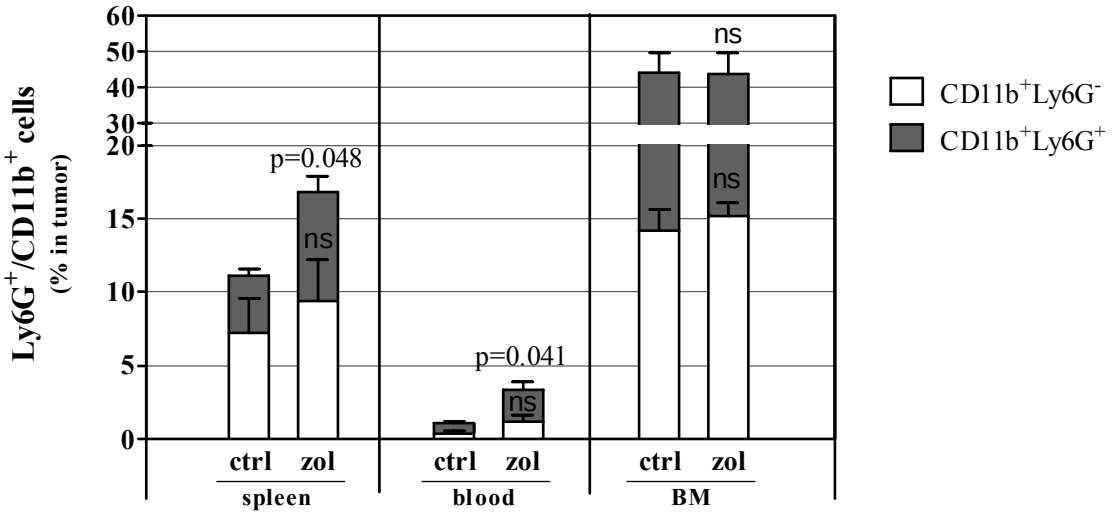

Figure S3. Effect of zoledronate treatment on abundance of myeloid cells in spleen, blood and bone marrow of LLC bearing mice. Spleen, blood and bone marrow samples were harvested from treated and untreated animals. Single cells suspensions were prepared as described in Material and Methods, stained and analyzed by flow cytometer. Spleen and blood samples from treated mice had a significantly higher percentage of CD11b+ Ly6G+ *neutrophils* compared to untreated controls. Experiments were done twice with at least 5 mice per group. Data represents ±SEM.

Supplementary Figure S4.

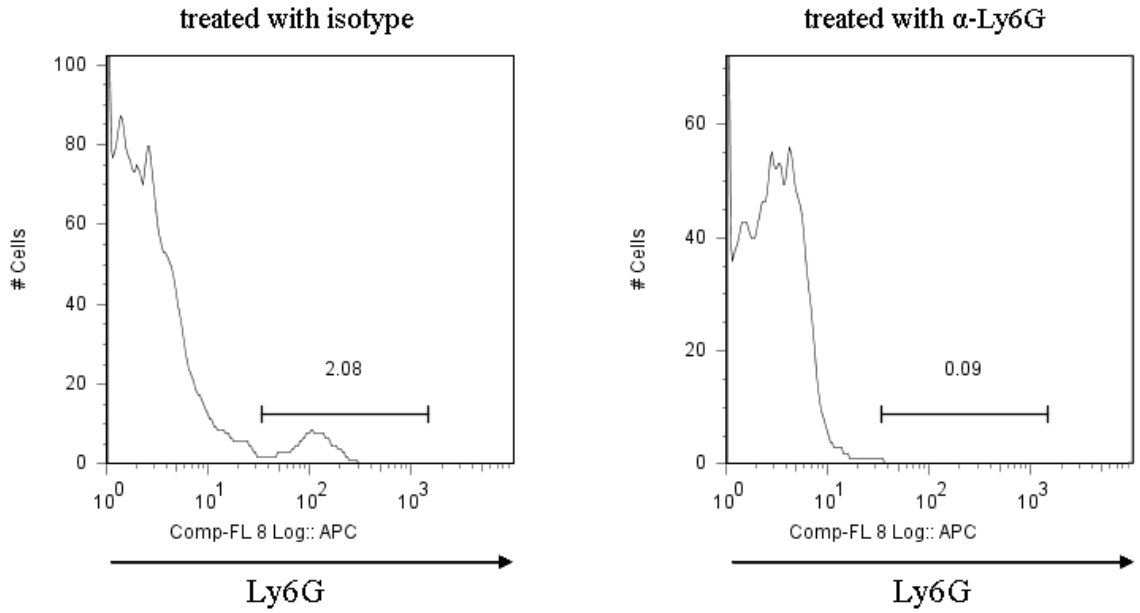

Figure S4: Efficient depletion of blood neutrophils by monoclonal α-Ly6G antibody,1A8. The Ab was administered as described in Materials and Maethods section. Blood samples were collected 24 hours after the last treatment by cardiac puncture. Cell depletion was confirmed by flow cytometry. 100 μg of purified antibody led to more than 90% depletion of Ly6G neutrophils in comparison to isotype control (rat IgG2b).

Supplementary Table 1. Sequences and annealing temperatures of primers used for real-time RT PCR are shown.

Name of primer	Sequence (5´-3´)	Annealing temperature
GAPDH	CAG GTT GTC TCC TGC GAC TT	55 ºC
	CCC TGT TGC TGT AGC CGT A	
β-actin	CTC TTC CAG CCT TCC TTC CTG	59 ºC
	GAA GCA TTT GCG GTG GAC GAT	
TNF-ά	CCT CCT GGC CAA CGG CAT GG	59 ºC
	AGG GGT GTC CTT GGG GCA GG	
IFN-γ	TCAAGTGGCATAGATGTGGAA	56 ºC
	CACTCGGATGAGCTCATTGA	
Arginase1	GAA GAA AGT GGT GCC ATG GAT AG	59 ºC
	CCC ATG AGT TCC ATG CTC AGA	
IL-12	Origene, MP206746	62 ºC
iNOS	Origene, MP208933	64 ºC
IL-10	Origene, MP206737	62 ºC
CD163	ATG GGT GGA CAC AGA ATG GTT	58 ºC
	CAG GAG CGT TAG TGA CAG CAG	
TGF-β	CCC CAC TGA TAC GCC TGA GT	57 ºC
	AGC CCT GTA TTC CGT TCT CTT	
VEGF	CCAGGAGGACCTTGTGTGAT	57 ºC
	GGGAAGGGAAGATGAGGAAG	
MHC-classII	GGC TCC TCA AGC GAC TGT GT	58 ºC
	GGG GCT GGA ATC TCA GGT TC	
CXCL1	CCG AAG TCA TAG CCA CAC TCA A	59 ºC
	GCA GTC TGT CTT CTT TCT CCG TTA C	
CXCL5	GGT CCA CAG TGC CCT ACG	59 ºC
	GCG AGT GCA TTC CGC TTA	
CXCL2	GAG CTT GAG TGT GAC GCC CCC AGG	60 ºC
	GTT AGC CTT GCC TTT GTT CAG TAT C	
CCL3	ACC ATG ACA CTC TGC AAC CA	60 ºC
	TCA GGC ATT CAG TTC CAG GT	

9. ACKNOWLEDGEMENT

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